MOLECULAR AND CELLULAR BIOLOGY, July 1994, p. 4759–4769
0270-7306/94/$04.00+0
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Vol. 14, No. 7

Disease-Activated Transcription Factor: Allergic Reactions in Human Skin Cause Nuclear Translocation of STAT-91 and Induce Synthesis of Keratin K17

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Received 19 October 1993/Returned for modification 2 December 1993/Accepted 14 April 1994

Epidermal keratinocytes have important immunologic functions, which is apparent during wound healing, in psoriasis, and in allergic and inflammatory reactions. In these processes, keratinocytes not only produce cytokines and growth factors that attract and affect lymphocytes but also respond to the polypeptide factors produced by the lymphocytes. Gamma interferon (IFN-γ) is one such signaling polypeptide. Its primary molecular effect is activation of specific transcription factors that regulate gene expression in target cells. In this work, we present a molecular mechanism of lymphocyte-keratinocyte signaling in the epidermis. We have induced cutaneous delayed-type hypersensitivity reactions that are associated with an accumulation of lymphocytes. These resulted in activation and nuclear translocation of STAT-91, the IFN-γ-activated transcription factor, in keratinocytes in vivo and subsequent induction of transcription of keratin K17. Within the promoter of the K17 keratin gene, we have identified and characterized a site that confers the responsiveness to IFN-γ and that binds the transcription factor STAT-91. Other keratin gene promoters tested were not induced by IFN-γ. These results characterize at the molecular level a signaling pathway produced by the infiltration of lymphocytes in skin and resulting in the specific alteration of gene expression in keratinocytes.

The role of epidermal keratinocytes in defense against mechanical injury and desiccation has been appreciated for a long time, but their role in immunological defense became apparent only recently, when it was realized that keratinocytes can produce a cornucopia of growth factors, chemoattractants, and cytokines (reviewed in reference 24). Furthermore, keratinocytes express receptors for many polypeptide factors, respond to autocrine stimulation, and also respond to the signals produced by the immune system (30). The importance of signaling between keratinocytes and lymphocytes is apparent in the cutaneous disorders that involve both of these cell types, including delayed-type hypersensitivity (DTH), psoriasis, and atopic dermatitis (21, 23, 27).

The most extensively studied signaling molecules of the immune system are the alpha, beta, and gamma interferons (IFN-α, IFN-β, and IFN-γ), a subset of cytokines originally described as factors that protect cells from viral infections (reviewed in references 35 and 41). Certain diseases are thought to be associated with high levels of IFN-γ in epidermis (27). Although the role of interferons in pathologic processes has not been clearly defined, they have been used recently in therapeutic trials for several dermatologic diseases, including those caused by viruses (12).

IFN-α and IFN-β share a cell surface receptor, whereas IFN-γ binds to a different receptor, and although their biological effects largely overlap, the effects of IFN-γ are distinct from those of IFN-α and IFN-β. Binding of interferon to its receptor initiates a cascade of events that include protein phosphorylation, but not necessarily production of second messengers (25). The cascade branches into a delta of transcription-activating pathways that induce multiple genes (22).

Interferons exert their effect on gene expression by activating transcription factors, which in turn bind to specific DNA sequences known as interferon-stimulated response elements and activate transcription of the nearby genes (17, 25, 31, 42). The primary IFN-α-responsive transcription factor, ISGF3, contains four proteins and is activated by phosphorylation of its tyrosines (14, 16, 40). One of its components, a protein of 91 kDa, termed STAT-91 (for signal-transducing activator of transcription), is specifically activated by IFN-γ. STAT-91 is found in the cytoplasm of unstimulated cells, but upon addition of IFN-γ to the culture medium, it is phosphorylated and translocates to the nucleus (7, 36), where it binds to the GAS (IFN-γ-activated sequence). Such sequences have been identified in regulated genes (26).

The human keratin K17 gene is among the genes specifically induced by IFN-γ in vitro (13). Keratins are a large group of cytoskeletal proteins specifically expressed in epithelial cells (38). They belong to two families, acidic (type I) and basic (type II), and coexpression of a member of each family is necessary for mutual costabilization and cytoskeletal assembly. As a rule, defined pairs of keratin proteins are coexpressed (e.g., K8 and K18 in simple epithelia, K5 and K14 in basal layers of multilayered epithelia, and K1 and K10 in differentiated layers of epidermis), although some cells express multiple pairs. K17 seems to be one of the exceptions to this rule because its specific coexpressed partner has never been found.

Keratin K17 is expressed in various epithelia (39), including myoepithelial cells, basal layers of transitional and pseudo-

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TABLE 1. Oligonucleotides used for PCR, cloning, and gel shift assays

Oligonucleotide	Sequence
Deletion constructs	
1 HR	TTTAAGCTTGGTGGCGGCGGCGGGA
460-XF	TTTTCTAGAGGTCACCTGACATTGC
350-XF	TTTTCTAGACTTCTCCCATATTAGG
310-XF	TTTTCTAGACCCGCCCAAATCACAG
280-XF	TTTTCTAGAAGCAGTGGCACGCACA
240-XF	TTTTCTAGATGGAGAAATGAGGCAA
200-XB	TTTTCTAGAGGATCCGGTCCCCAGCCACCTGGGAG
160-XB	TTTTCTAGAGGATCCATGAAAGCCCAGGGGAACGG
130-XB	TTTTCTAGAGGATCCGCCTGTTGTAATCGTACGCC
Functional gene constructs	
F350 BAM	TTTGGATCCAAATCACAGGTGACGGGCCTCAG
F460 BAM	TTTGGATCCCATTGCAACCCATTTCCCA
RMET	TTTGAATTCCAGGCCGCCAGCAGATCCCA
R-5′-LDR	TTTGAATTCGGAGAAGGGCTGGAGAGG
R-145-Sal	TTTGTCGACCAGGCCGCCAGCAGATCCCA
R-286-Sal	TTTGTCGACGGAGAAGGGCTGGAGAGG
Enhancer constructs	
LN-460-F	TTTGGATCCGAGCTCGGTCACCTGACATTGCAACCGATT
LN-200-R	TTTAGATCTCCCTCCCCACCCATCATCAG
IFNG-F	TTTGGATCCCCGCGGATTTGGAGAAATGAGGCAAATTCCTGATGATGGGTRGGGGAGGGAGATCTAAA
IFNG-R	TTTAGATCTCCCTCCCCACCCATCATCAGGAATTTGCCTCATTTCTCCAAATCCGCGGGGATCCAAA
3*GAS-F	TTGGATCCCCGCGGATTTGGAGAAATGAGGCAAAGGATTTGGAGAAATGAGGCAAAGGATTTGGAGAAATGAGGCAAAGATCTAAA
3*GAS-R	TTAGATCTTTGCCTCATTTCTCCAAATCCTTTGCCTCATTTCTCCAAATCCTTTGCCTCATTTCTCCAAATCCGCGGGGATCCAAA
Mutated constructs	
340BglII-R	TTTAGATCTAGAAGAGGAGAA
340BglII-F	TTTAGATCTTAGGTCATGGGAAA
340PvuII-R	TTTCAGCTGATGACCTAATACCGGAGAAG
340PvuII-R	TTTCAGCTGGCATAGCTGGAGGGCCCGC
240EcoRI-R	TTTGAATTCTCCAAATCCTCGTGCTGAGTGC
240EcoRI-F	TTTGAATTCAGGCAAATTCCTGATGATGG
240PvuII-R	TTTCAGCTGTCCTCGTGCTGAGTGCC
240PvuII-F	TTTCAGCTGGAAATGAGGCAAATTCC

stratified epithelia of the respiratory and urinary tracts, and early developmental stages of stratified epithelia. Common characteristics of these cells are contractility and/or frequent changes in shape. Normal adult epidermis does not contain keratin K17. Certain pathological states, including psoriasis as well as benign and malignant neoplasms, lead to K17 expression. Indeed, expression of K17 has been used to evaluate the course of treatment of psoriatic patients (9).

Because of the complexities and importance of the interaction of the immune system with the epithelial component of the epidermis, we decided to investigate the molecular effects of IFN-γ on transcription of epidermal keratin genes, and specifically of the keratin K17 gene, both in vivo and in vitro. We found that we could experimentally induce in vivo expression of keratin K17 by causing a DTH inflammatory reaction characterized by substantial infiltration of lymphocytes that produce IFN-γ (21). In affected epidermis, we found transcription factor STAT-91 in the nuclei of keratinocytes. In contrast, STAT-91 is cytoplasmic in unaffected, healthy skin.

Having assembled a large collection of DNA constructs in which upstream sequences of keratin genes regulate expression of the chloramphenicol acetyltransferase (CAT) reporter gene, we transfected these constructs into epidermal keratinocytes in vitro and then grew the cells in the presence or absence of IFN-γ. We found that IFN-γ strongly and specifically induced the promoter of the K17 gene. No other keratin gene construct was induced. Furthermore, we have characterized the GAS of the K17 gene and compared it with previously reported GASs. We have also demonstrated that the K17 gene GAS binds activated STAT-91 and that the p91 protein component of ISGF3 is included in the STAT-91 complex that binds to the K17 gene upstream sequence.

Changes in transcription factors have been well characterized in oncogenesis; indeed, many proto-oncogenes are transcription factors. However changes in transcription factors due to nononcogenic diseases have not been described until now. Our results define at the molecular level how IFN-γ regulates expression of the K17 keratin gene and provide a means for analysis of the molecular interactions between the immune system and the epidermis, interactions that are important in pathological skin processes.

MATERIALS AND METHODS

DNA constructs. The keratin promoter-containing plasmids and the control plasmids pSV2CAT and pRSVZ have been described previously (18–20). Additional constructs were prepared by PCR with *Thermus aquaticus* DNA polymerase under conditions suggested by the manufacturer (Perkin-Elmer Cetus). DNA primers were synthesized on a Pharmacia Gene-Plus synthesizer. They are listed in Table 1. Amplified DNAs were digested with appropriate restriction enzymes and ligated into similarly digested and gel-purified pGCAT vector. The

sequences of the DNAs inserted into the pGCAT vector were confirmed by the dideoxy plasmid sequencing method.

K17CAT was engineered by using oligonucleotides K17F and K17R in PCR with λ phage 2F12 as the template (33). Note that the K17 gene in 2F12 derives from the human keratin pseudogene locus. Our initial experiments were conducted with the pseudogene DNA, and the deletion constructs presented in Fig. 5B contain pseudogene sequences. In the meantime, the sequence of the functional gene became available (39), and with this sequence as a guide, we designed oligonucleotides and cloned the promoter of the functional K17 gene. Within the 5′ leader region reported, the sequence of our functional K17 gene clone matches, with a single base pair difference, the sequence of the functional gene and differs from the sequences of the two pseudogenes. The results in all figures except 5B were obtained with the functional sequences.

To create the deletions of the K17 promoter we used PCR with K17CAT as a template and primers containing an *Xba*I, *Pst*I, or *Hin*dIII site. The proximal (reverse) primer starts just upstream of the ATG translation initiation codon.

Mutagenesis was performed by using a two-round PCR (3). The target sequence is converted into a restriction site. Briefly, in the first round, two PCR products are created, from the common restriction site to the outlying primers. The two products are cut at the restriction site and ligated, creating a long template for the second round of PCR, using just the outlying primers. The product of the second round is cloned into a CAT-containing vector, and the new restriction site serves as a convenient marker for screening (for details, see reference 3).

Synthetic DNA corresponding to the downstream GAS site and PCR-amplified DNA containing both putative GAS sites were cloned into the enhancer trap pCAT-Promoter vector (Promega), using either its *Bgl*II or its *Bam*HI cloning site. The synthesized DNAs contained either one or three GAS sites in tandem, bracketed by restriction sites that facilitate cloning (Table 1). The CAT activity of the resulting plasmid was compared with that of control plasmid pCAT-Promoter (Promega).

Gel retardation. Nuclear extract (approximately 5 μg of protein) from IFN-γ-treated or control FS2 cells (37) was first incubated for 15 min on ice with or without a 200-fold molar excess of double-stranded DNAs used as unlabeled competitors in 25 μl of binding buffer containing 2 μg of poly(dI-dC), 10% glycerol, 5 mM $MgCl_2$, 1 mM dithiothreitol, 100 mM NaCl, 20 mM Tris-HCl (pH 8), 2% polyvinyl alcohol, 0.1 mM EDTA, and 4 mM spermidine. The probe was end labeled with the Klenow fragment and [α-^{32}P]dCTP (40 μCi per reaction) and purified by gel filtration on Sephadex G-50 columns (Excellulose GF5; Pierce). Oligonucleotide probe (80,000 cpm, 1 to 10 pg) was then added, and the mixture was incubated for an additional 15 min on ice. Probe bound to nuclear protein was resolved from free probe through a 4% polyacrylamide gel (acrylamide-bisacrylamide, 29:1). After drying, gels were autoradiographed overnight at −70°C on XAR 5 film (Kodak) with screen intensifiers. For supershift experiments, the p91-specific antibody (34) was added either prior to addition of the radioactive probe or after the incubation, in which case the samples were left on ice for an additional 20 min. The two protocols gave identical results. The antibody was used diluted 1:120 in the supershift.

Cells and transfection. HeLa S3 cells were maintained in Dulbecco modified Eagle's medium supplemented with 10% fetal calf serum. Normal human foreskin epidermal keratinocytes were purchased from Clonetics or were a generous gift from M. Simon. They were grown in defined serum-free keratinocyte medium supplemented with epidermal growth factor (EGF) and bovine pituitary extract (SFM; Gibco). Cells were expanded through two 1:4 passages before transfection.

HeLa S3 cells were transfected by using $Ca_3(PO_4)_2$ precipitation. Keratinocytes were transfected at 80% confluency, using Polybrene and dimethyl sulfoxide shock as previously described (19). Confluent cultures (Fig. 2C) were transfected 1 day after the plates were completely covered with cells. In all experiments, plasmid pRSVZ was included as an internal control for the efficiency of transfection.

All DNAs used in transfections were purified by using a Magic Megapreps DNA purification system (Promega). At 48 h after transfection, cells were washed twice with phosphate-buffered saline (PBS) and harvested by scraping. Procedures for cell disruption by repeated freeze-thaw cycles, as well as CAT and β-galactosidase assays, have been described elsewhere (18, 20). Briefly, the substrate solution contained 6 mg of *o*-nitrophenyl-β-D-galactoside (Sigma) freshly dissolved in PM2 buffer (33 mM NaH_2PO_4, 66 mM Na_2HPO_4, 0.1 mM $MnCl_2$, 2 mM $MgSO_4$, 40 mM mercaptoethanol). The reaction mixture contained 100 μl of substrate solution, 300 μl of PM2 buffer, and 50 μl of cell extract. It was incubated at 37°C until development of a yellow color was obvious, usually 0.5 to 1 h. The time of the reaction was recorded, and the reaction was stopped by addition of 0.4 ml of 1 M Na_2CO_3. The optical density at 420 nm (OD_{420}) was measured on a UV spectrophotometer (Gilford). β-Galactosidase activity was calculated by the formula (1,000 × OD_{420}/micrograms of pRSVZ DNA) × (150/microliters of extract used) × (60/minutes of reaction time).

The CAT reaction mixture contained 69 μl of 1 M Tris HCl (pH 7.8), 1 μl of [^{14}C]chloramphenicol (40 to 50 mCi/mmol; New England Nuclear), 20 μl of 4 mM acetyl coenzyme A (1.6 mg freshly dissolved in 0.5 ml of cold H_2O), cell extract, and water to bring the total reaction volume to 150 μl. After incubation at 37°C for 1 h, the mixture was extracted into 1 ml of ethyl acetate, phases were separated by brief centrifugation, the organic layer was transferred to a new tube, and the solvent was evaporated. The residue was dissolved in 30 μl of ethyl acetate and separated by silica gel thin-layer chromatography in chloroform-methanol (95:5). The plates were exposed to X-ray film for 12 to 24 h, and the radioactive spots were identified, excised from the plates, and counted. The conversion of chloramphenicol to its monoacetylated derivative was kept below 50% by varying the amount of extract or the duration of the reaction. The CAT activity for a 1-h reaction was calculated by the formula [cpm (acetylated chloramphenicol)/cpm (acetylated chloramphenicol + chloramphenicol)] × (150 μl/50 μl) × 1/micrograms of CAT plasmid).

All CAT values were normalized for transfection efficiency by calculating the ratio of CAT activity to β-galactosidase activity in each transfected plate. Each transfection experiment was performed three or more times, with each datum point resulting from duplicate or triplicate transfections.

Immunohistology. The forearms of healthy volunteers were injected with 0.1 ml of mumps antigen skin test (Connaught). After 48 h, 4-mm punch biopsy samples were taken from the mumps antigen injection site (DTH site) and from a normal uninjected adjacent site on each volunteer. These samples were embedded in Tissue Tek OCT compound (Miles Scientific), frozen in liquid nitrogen, and stored at −70°C. Frozen sections were cut from 6 to 8 μm (Fung Frigocut 2,800 E cryostat) and collected onto gelatin-coated slides.

The sections were stained by the standard immunofluorescent staining protocol (28). The primary antibodies used were mouse monoclonal antibody CK17 (IBL Research Products

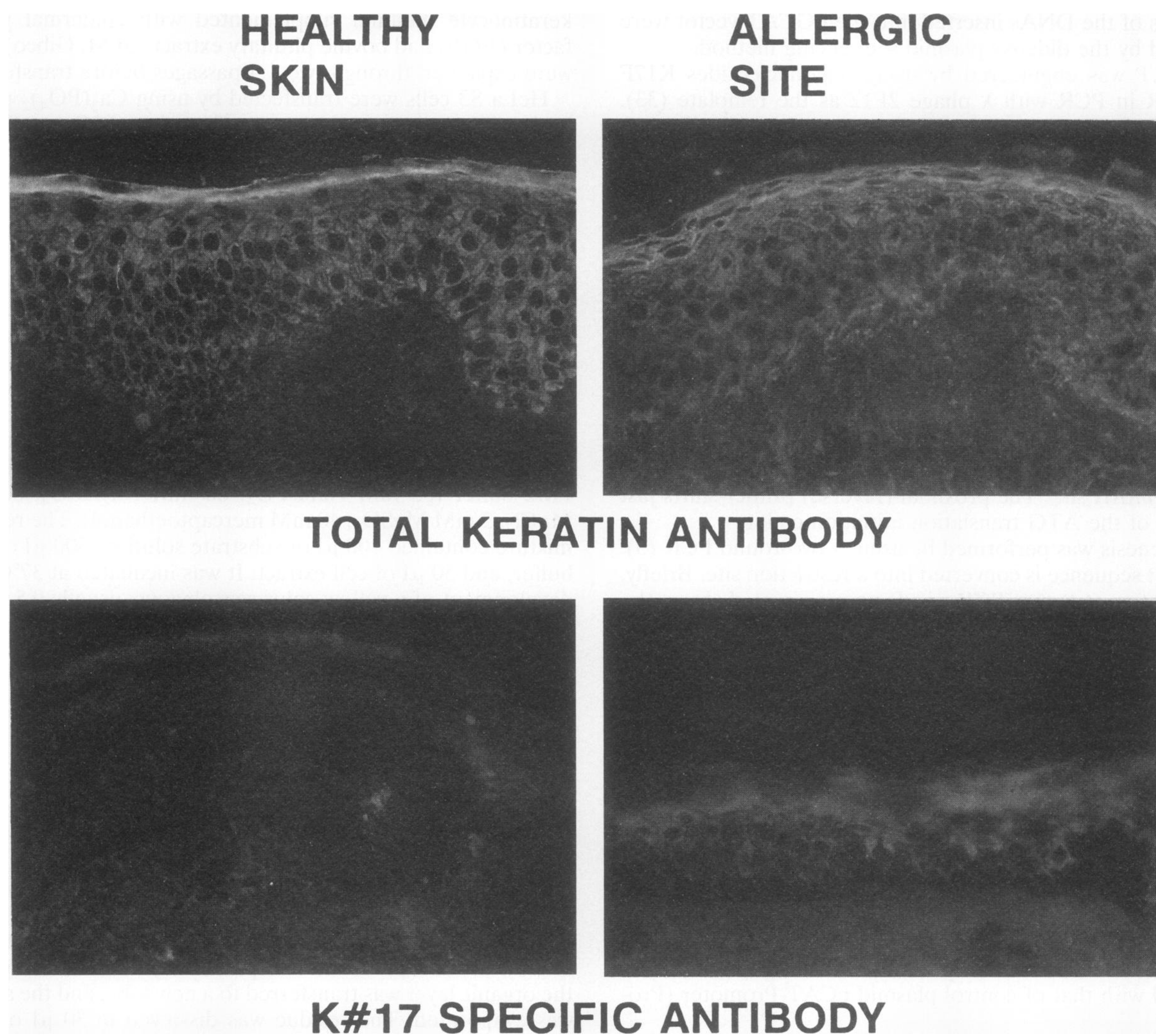

FIG. 1. Induction of keratin K17 expression *in vivo*. A DTH reaction was elicited with mumps vaccine, and a biopsy sample (right) was compared with the control biopsy sample of a healthy site (left) from the same volunteer. Immunofluorescence with general antikeratin antibody AE3 (top) and with a K17-specific antibody (bottom) is shown. Note that only the affected site is reactive with the K17 antibody.

Corp.), p91 rabbit antibody (34), and AE3, a general antikeratin monoclonal antibody (gift of T.-T. Sun). As the secondary antibodies, anti-mouse immunoglobulin G-fluorescein isothiocyanate conjugate adsorbed with human serum proteins (for CK17 and AE3) and anti-rabbit immunoglobulin G-fluorescein isothiocyanate conjugate adsorbed with human serum proteins (for p91; both from Sigma Immuno Chemicals) were used.

Human epidermal keratinocytes were grown to passage 4, plated on glass coverslips, and grown for 24 h in normal medium (SFM; Gibco). The keratinocytes were then treated with IFN-γ (250 U/ml) for various lengths of time, ranging from 1 min to 4 h. Several coverslips with keratinocytes were left untreated as controls. The cells were washed twice with PBS and then fixed with methanol-acetone (1:1) for 5 min. After drying, they were stored at $-20°C$. The coverslips were stained with the p91-specific and secondary antibodies as described above.

Protein purification and Western blotting (immunoblotting). Cell pellets were homogenized in buffer containing 25 mM Tris-HCl (pH 7.6), 1 mM EGTA, 1 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride, antipain (50 μg/ml), aprotinin (2 μg/ml), and pepstatin A (1 μg/ml). The homogenate was centrifuged at $10,000 \times g$ for 20 min, and the insoluble protein pellets were again homogenized and centrifuged as described above. The keratin proteins were obtained by extracting the insoluble pellets with 25 mM Tris-HCl–9 M urea. Protein concentrations were determined by a Bio-Rad assay. Gel electrophoresis and immunoblot analysis, using the same K17-specific antibody, were performed as described previously (5).

In ^{35}S labeling experiments, HeLa cells at 90% confluency were washed and transferred to methionine-free Dulbecco modified Eagle's medium. Ten minutes after addition of 50 μCi of [^{35}S]methionine (ICN) per ml, the cells were rinsed and harvested, and the cytoskeletal proteins were isolated as described above. IFN-γ (250 U/ml) was added 2 h before the addition of radioactivity.

RESULTS

In vivo induction of K17 synthesis. Healthy human interfollicular epidermis does not contain keratin K17. However, in psoriasis, an epidermal proliferative disease with important

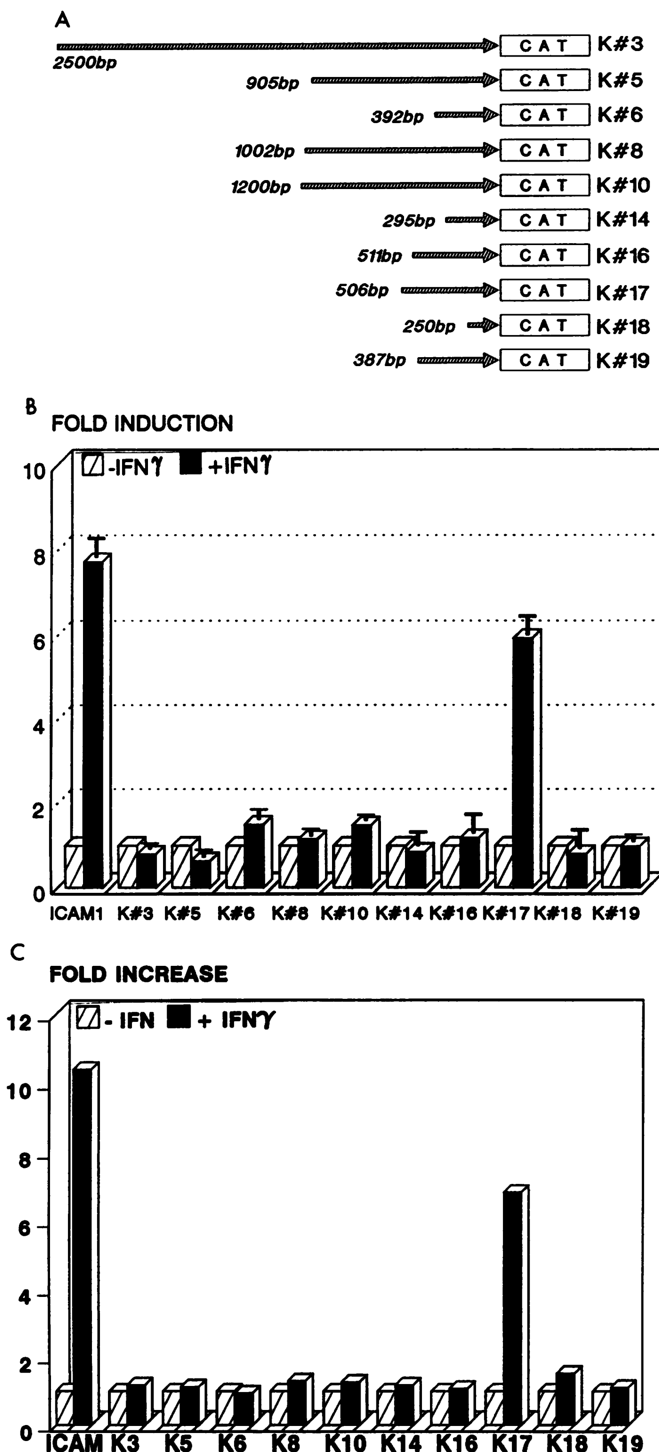

FIG. 2. IFN-γ induces transcription from the K17 promoter. Human epidermal keratinocytes were transfected with constructs containing the gene promoters indicated and grown in the presence or absence of IFN-γ. (A) Constructs used in the transfection. Arrows represent the keratin promoter DNAs drawn approximately to scale, with numbers indicating the lengths. (B and C) Results with preconfluent and postconfluent keratinocytes, respectively. Each construct was transfected between three and six times in duplicate. The error bars represent the differences between the duplicates.

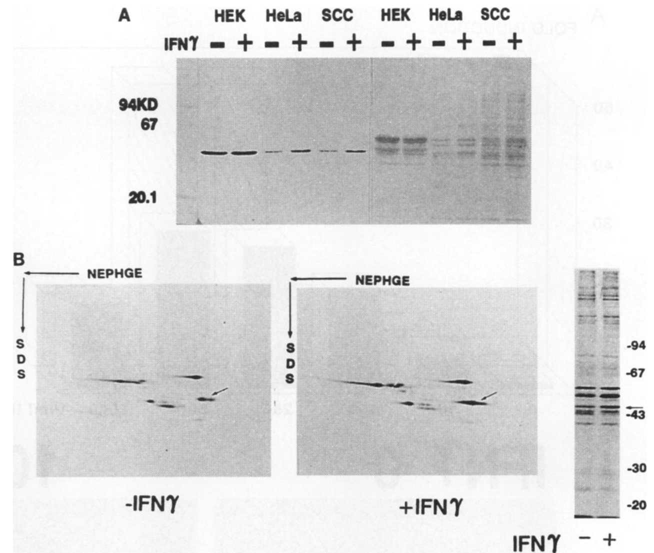

FIG. 3. Induction of endogenous keratin K17 synthesis by IFN-γ. (A) Cytoskeletal proteins of IFN-γ-treated and untreated cells of three different epithelial cell types were resolved on polyacrylamide gels and stained (right). Note that the preexisting K17 protein in the untreated cells as well as the cytoskeletal proteins of similar mobility obscure the induction of keratin K17 by IFN-γ. In immunoblots (left), the K17-specific antibody highlights the higher level of K17 in IFN-γ-treated cells. (B) Keratinocytes were pulse-labeled with ^{35}S; the cytoskeletal proteins were purified as described above, resolved on polyacrylamide gels, and autoradiographed (right). The two-dimensional gels (left) show a more intensive labeling of the keratin K17 spot, marked with arrow. NEPHGE, non-equilibrium pH gradient gel electrophoresis; SDS, sodium dodecyl sulfate gel electrophoresis.

immunological components, K17 is expressed (8). To determine whether K17 expression is peculiar to psoriasis or more generally a consequence of cutaneous lymphocytic infiltration, we induced, in healthy laboratory volunteers, a strong allergic reaction by subcutaneous injection of mumps antigens. Forty-eight hours after injection, a biopsy was taken from the inflamed area and from adjacent noninflamed control skin. The biopsy samples were sectioned and stained with a general antikeratin antibody and with an antibody specific for keratin K17. As expected, control skin did not contain keratin K17, but the inflammation resulted in substantial expression of keratin K17 (Fig. 1). K17 is found in the suprabasal layers; the basal layer is generally devoid of K17. This keratin appears in filamentous cytoskeletal structures typical of intermediate filaments in these cells; presumably it is integrated into the preexisting keratin network. Only the keratinocytes in the inflamed area contain K17; adjacent regions do not.

IFN-γ induces transcription from the K17 gene promoter. Lymphocytic infiltrates can produce IFN-γ, and earlier reports indicated that IFN-γ increases the level of keratin K17 mRNA (13). To determine whether the effect of IFN-γ is at the level of transcription initiation, we created a DNA construct in which the upstream sequence of the K17 gene drives the expression of the CAT reporter gene. This construct was transfected into primary human epidermal keratinocytes, which were then cultured in the presence or absence of IFN-γ. As a positive control, we used a construct containing a segment of the human ICAM-1 gene that has been shown previously to be regulated by IFN-γ (1, 8, 10). We found that IFN-γ strongly induces transcription from the K17 gene promoter (Fig. 2).

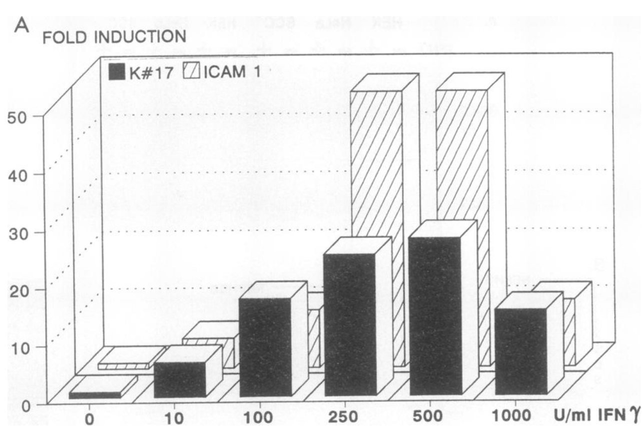

FIG. 4. The effect of IFN-γ is concentration dependent. The transfected keratinocytes were grown in the presence of various amounts of IFN-γ. (A) Results of CAT assays. The optima for both K17 and ICAM-1 induction are between 250 and 500 U/ml. (B) Changes in the keratinocyte phenotype. With increasing concentration of IFN-γ, long processes extend from the cells, the intercellular borders become less refractile, and many cells detach from the substrate.

The effect of IFN-γ on the K17 promoter is not limited to keratinocytes; it is also demonstrable in HeLa S3 cells (data not shown).

To assess the effect of IFN-γ on the expression of other keratin genes, we used a rather extensive collection of DNA constructs in which the upstream sequences of human keratin genes drive expression of CAT reporter gene (20) and transfected them into cultured human keratinocytes. The transfected keratinocytes were grown in the presence or absence of IFN-γ in the medium. As shown in Fig. 2, addition of IFN-γ strongly and specifically induces transcription only from the DNAs of the ICAM-1 and K17 genes.

The upstream sequences tested in this experiment included those for differentiation-specific keratin K3 and K10 genes,

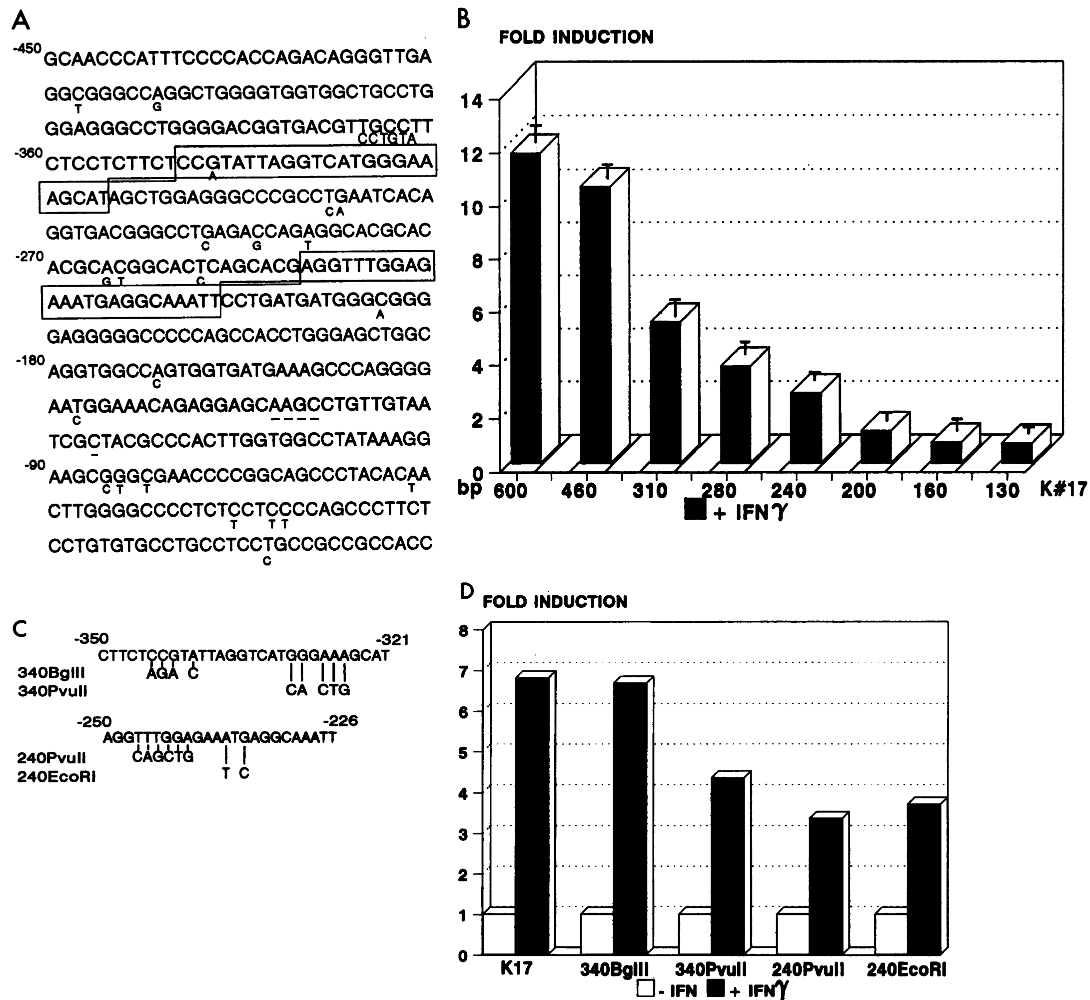

FIG. 5. Mapping of GASs within the K17 promoter. (A) Sequence of the functional K17 keratin gene promoter. The differences in the previously published pseudogene sequence (33) are shown in smaller print below the sequence. Deletion analysis indicated that the two boxed segments probably contain GASs. (B) IFN-γ inducibility of the deletion constructs. The two largest constructs are equally induced; the construct containing 240 bp of the K17 promoter is inducible, but the construct containing only 200 bp is not. (C) Mutated sequences in the two GASs. (D) Effects of mutations on IFN-γ responsiveness. Three of the mutations impair IFN-γ responsiveness, but the mutation in 340BglII does not.

basal layer-specific K5 and K14 genes, activation-specific K6 and K16 genes, simple epithelial cell-specific K8 and K18 genes, and the keratin K19 gene. The various constructs have different basal, unstimulated activities, but whereas ICAM and K17 constructs are strongly induced by IFN-γ, those of other keratin genes are not affected. From these data, we conclude that of the keratin genes tested, the K17 gene is the only one induced by IFN-γ.

When they reach confluence, cultured keratinocytes change their phenotype. They become more difficult to transfect, but more relevantly, they initiate a program of terminal differentiation, which in culture they do not complete. It has been proposed, on the basis of intracellular aggregation of keratin intermediate filaments, that IFN-γ promotes differentiation of A431 cells (4); therefore, we tested the effects of IFN-γ in postconfluent keratinocytes. Even in postconfluent cells, IFN-γ strongly and specifically induced only the promoter of the K17 keratin gene (Fig. 2C). The promoters of the K3 and K10 genes, markers of differentiation, were unaffected.

Cultured keratinocytes and other epithelial cell types produce a significant amount of keratin K17 even without IFN-γ. Culture conditions may contain inducers of K17 that are as yet unidentified, although EGF is not one of them (20). Transcription factors AP2 and Sp1 play a role in expression of the K17 gene (29). IFN-γ increases the endogenous expression of K17. If the cells are pulse-labeled with [^{35}S]methionine during the treatment with IFN-γ, although the total protein levels do not change, the label preferentially accumulates in K17 protein (Fig. 3).

The induction by IFN-γ is concentration dependent. The strongest effect of IFN-γ on K17 and ICAM gene transcription is seen at concentrations of 250 and 500 U/ml (Fig. 4). Within this concentration range, IFN-γ profoundly affects the keratinocyte phenotype (Fig. 4B). Without IFN-γ, keratinocytes have a tightly packed, cuboidal, cobblestone-like appearance with clearly marked cell boundaries. Even at the lowest concentration of IFN-γ tested, 10 U/ml, the attachment of transfected cells to the substrate weakens. At higher concentrations, the intercellular bonds also weaken and long processes extend from the cells. At the highest concentra-

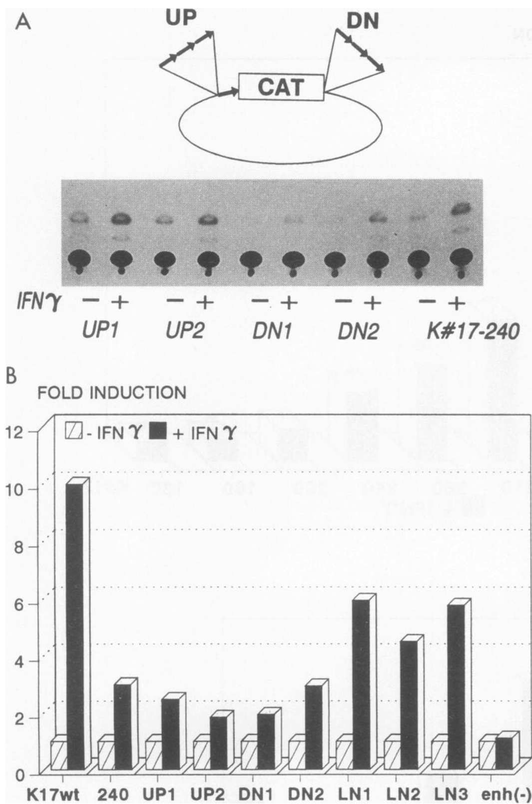

FIG. 6. The GAS can confer IFN-γ responsiveness to a heterologous promoter. (A) Three copies of the downstream GAS confer IFN-γ responsiveness to an enhancer trap construct, both upstream (UP1 and UP2) and downstream (DN1 and DN2) from the CAT gene and in both the correct (UP1 and DN1) and opposite (UP2 and DN2) orientations. (B) Quantitative presentation of the results indicates that one copy of the GAS is nonfunctional but three copies are functional. A single copy of longer fragment, bp 200 to 460, which contains both GASs, confers strong IFN-γ responsiveness, both upstream (LN1 and LN2) and downstream (LN3) from the CAT gene. K17wt, wild-type K17; enh(−), enhancer deficient.

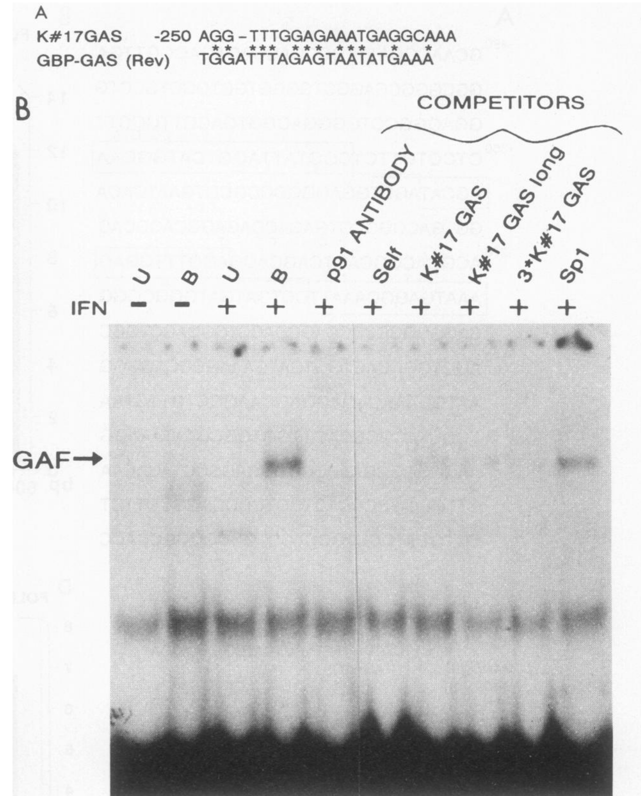

FIG. 7. STAT-91 binds to the GAS in vitro. (A) Sequence comparison between K17 and GBP GASs. Note that the GBP GAS is shown in reverse (Rev) orientation. (B) Gel retardation assay using the GAS from the Ly-6E gene and the K17 promoter GAS as a competitor. IFN-γ-induced and uninduced cells were the sources of nuclear proteins. U and B stand for unbound (i.e., without nuclear extract) and protein-bound DNA, respectively. Antibody to the p91 protein creates a supershift. Specific DNA competitors contain either the unlabeled probe (Self), a 20-bp (K#17 GAS) or 50-bp (K#17 GAS long) sequence, or three tandem copies of the 20-bp sequence (3*K#17GAS) shown in Table 1. Nonspecific DNA was the Sp1 consensus element. A 200-fold excess of unlabeled competitors was used. The arrow marks the STAT-91-specific retarded band. GAF, IFN-γ-activated factor.

tions tested, the majority of the transfected cells are detached. Those remaining either have extended processes or are flattened with barely visible intercellular boundaries (Fig. 4B).

Identification of the GAS. Our initial experiments used a promoter derived from a K17 pseudogene (33). During these studies, the sequence of the functional K17 cDNA was published (39), and we used it to clone the functional gene of the promoter (Fig. 5A). The functional gene has the same response to IFN-γ as the promoter of the pseudogene does (Fig. 2).

To identify the DNA sequence elements responsible for the effects of IFN-γ on the K17 promoter, we created a set of 5′ deletions and tested them both in keratinocytes and in HeLa S3 cells (Fig. 5B). The construct containing 310 bp is induced only half as much as the two largest constructs, which suggests that there is a GAS upstream from the −310 position. As additional sequences are deleted, the inducibility gradually declines. Two constructs, containing 240 and 210 bp, have the same basal, uninduced promoter activity. However, whereas the construct containing 240 bp is inducible by IFN-γ, the construct containing 210 bp is not. This finding localizes another GAS within the −210 to −240 DNA segment. To determine whether these two segments are indeed GASs, we introduced point mutations into the keratin K17 gene promoter DNA. Of the two mutations near bp −340, one (340PvuII) is within a GAS because it reduced IFN-γ responsiveness; the other (340BglII) did not. Both mutations near bp −240 caused a significant (40 to 50%) reduction in the effect of IFN-γ, confirming that these sequences are indeed GASs (Fig. 5D).

If these sequences are sufficient to confer responsiveness to IFN-γ, then it should be possible to transfer the responsiveness to a heterologous promoter. To test this possibility, we cloned the downstream GAS of the K17 promoter into an enhancer trap vector. This vector has the simian virus 40 promoter in front of the CAT gene but is missing the enhancer and is therefore transcriptionally inactive. Insertion of a single copy of the GAS, in either orientation, both upstream and downstream from the CAT gene had no effect. However, when we inserted three copies of the GAS in tandem, we observed IFN-γ responsiveness (Fig. 6). Without IFN-γ, the insert did not enhance transcription; i.e., the GAS is not intrinsically an enhancer. However, in the presence of IFN-γ, transcription

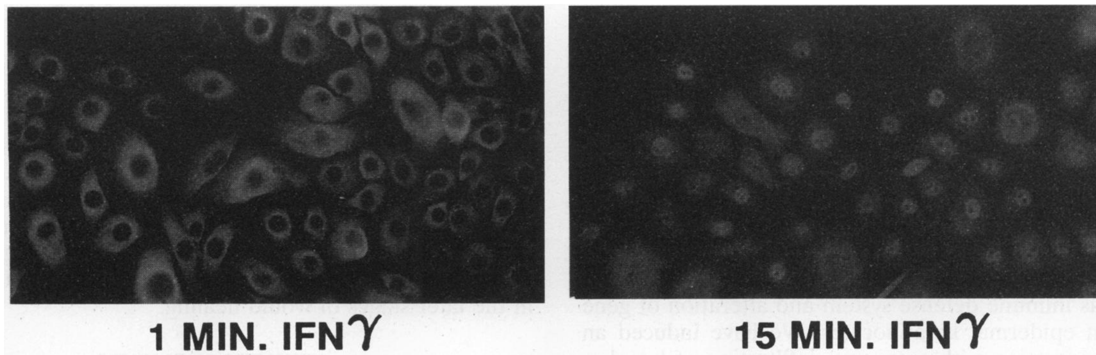

FIG. 8. STAT-91 translocates into the nucleus of IFN-γ-treated keratinocytes. Immunohistologic staining with a p91-specific antibody shows diffuse cytoplasmic localization of the p91 epitope 1 min after IFN-γ addition but nuclear localization after 15 min in the IFN-γ-treated cultured keratinocytes.

was significantly increased. Three copies of the GAS confer responsiveness to IFN-γ in either orientation, both upstream and downstream from the transcription initiation site (Fig. 6).

The downstream GAS interacts with the surrounding sequences because a single copy is sufficient to confer IFN-γ responsiveness to the K17 keratin gene promoter (e.g., constructs 200 and 240 in Fig. 5B), but a heterologous promoter requires multiple copies, a situation similar to that for the guanylate-binding protein (GBP) gene (6). A longer segment that contains both the upstream and the downstream GAS conferred strong IFN-γ responsiveness; a single copy of the longer segment was sufficient for this effect (Fig. 6).

The GAS binds STAT-91 in vitro. Perusal of the functional K17 gene promoter sequence identified a segment similar to that of the GAS of the GBP gene, albeit in the reverse orientation, near bp −240 (Fig. 7A). To determine whether this segment can bind the same STAT-91 as the previously characterized GAS of the Ly-6A/E gene (22), we used it as a competitor in gel shift experiments (Fig. 7B). Nuclear extracts of cells grown in the presence and absence of IFN-γ identify the shifted band specific for the induction by IFN-γ. Furthermore, this band is supershifted by the antibodies prepared against the 91-kDa STAT-91 protein (22, 36). All oligonucleotides that contain the K17 promoter downstream GAS sequence compete for the same protein in a gel shift assay, which demonstrates that STAT-91 binds the K17 gene GAS. However, we could not demonstrate directly the binding of STAT-91 to the K17 GAS in gel shift assays, which probably means that the binding of STAT-91 to the K17 GAS is weaker than binding to the Ly-6A/E GAS. The competition is specific because other oligonucleotides, e.g., the Sp1 consensus site DNA shown in Fig. 7, do not compete. These experiments suggest that the GAS in the K17 promoter binds the previously characterized p91 STAT-91 transcription factor.

IFN-γ causes accumulation of STAT-91 in the nuclei of keratinocytes. STAT-91 resides in the cytoplasm of HeLa and FS2 cell lines in the absence of IFN-γ but moves into the nucleus upon activation (36). The same process occurs in primary cultures of human epidermal keratinocytes. Untreated keratinocytes stained with a p91-specific antibody show diffuse cytoplasmic immunofluorescence. The nuclei are unstained (Fig. 8). If treated with IFN-γ, within 15 min, the nuclei of keratinocytes are brightly stained, which indicates that in this period the p91 was activated and entered the nuclei. The mechanism described for immortalized cell lines is operational in the primary cultures of human epidermal keratinocytes as well.

To test whether nuclear translocation of STAT-91 occurs in

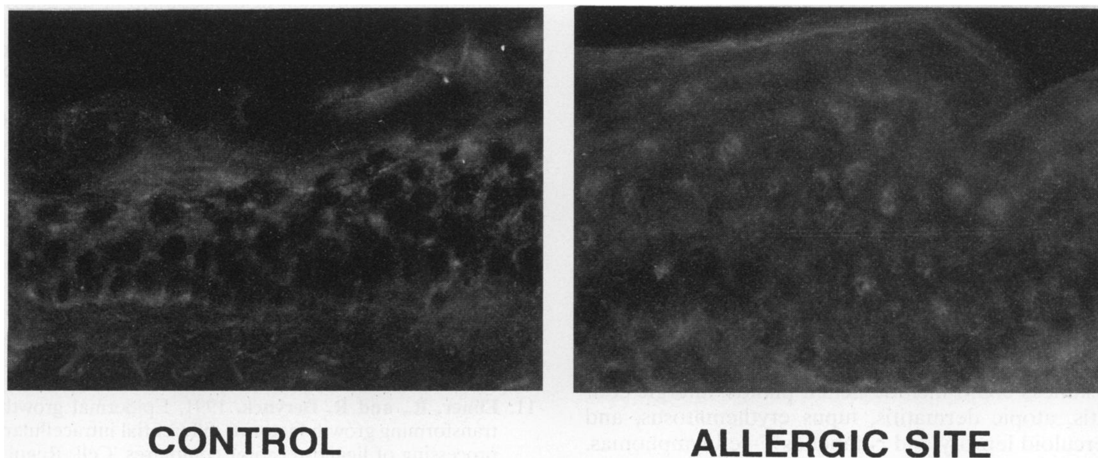

FIG. 9. Inflammation causes nuclear translocation of STAT-91 in vivo. Sections of healthy skin (left) and of a DTH site biopsy sample (right) were stained with a p91-specific antibody. In the healthy keratinocytes, p91 is cytoplasmic while the nuclei are devoid of p91, which gives the epidermis a Swiss cheese-like appearance similar to the cytoplasmic staining with antikeratin antibody (Fig. 1). In contrast, in the inflamed area, p91 accumulates in the nuclei, which appear as bright dots, leaving the cytoplasms depleted.

vivo, we examined sections of healthy human skin and of DTH reaction biopsy samples. As shown in Fig. 9, the location of STAT-91 in healthy epidermis was exclusively cytoplasmic. The nuclei were unstained. In the DTH biopsy sample, however, the protein was found largely in the nucleus. The same mechanism, activation and nuclear translocation, is also operational in vivo.

DISCUSSION

Our results provide a molecular link between activation of the cutaneous immune defense system and alteration of gene expression in epidermal keratinocytes. We have induced an inflammatory reaction with cutaneous infiltration of lymphocytes and shown that this infiltration results in nuclear translocation of the IFN-γ-responsive transcription factor STAT-91, which in turn can induce transcription of the keratin K17 gene. We have characterized the GAS in the promoter of the K17 gene and demonstrated its interaction with STAT-91.

Interestingly, EGF can also cause phosphorylation and activation of STAT-91 (15, 32, 37). In our experiments, keratinocytes were grown in the presence of EGF, but the long-term effects of EGF may be quite different from the transient pulse of EGF addition. Indeed, EGF and to a lesser extent transforming growth factor α cause internalization and degradation of their receptors, which attenuates the response to EGF and transforming growth factor α (11). However, even a transient pulse of EGF does not induce synthesis of keratin K17, but instead it induces synthesis of keratins K6 and K16 (20); these keratins are not induced by IFN-γ (Fig. 2). Thus, although multiple signaling pathways may partly converge through STAT-91, the specificity of the extracellular signals is maintained in the nucleus.

Results from several sources have established that lymphocytes induce expression of HLA-DR and ICAM-1 cell surface markers in keratinocytes and that IFN-γ mediates these signals from lymphocytes to keratinocytes (1, 9, 10). Taking these findings further, we have shown that IFN-γ causes activation of STAT-91 and its translocation into the nucleus as the next step of the signaling pathway. The translocation occurs both in cultured cells and in skin in vivo. Immunofluorescence using STAT-91-specific antibodies shows diffuse cytoplasmic staining before, and concentrated nuclear staining after, treatment with IFN-γ or development of a DTH reaction.

In the subsequent step of IFN-γ signaling, activated STAT-91 binds a specific site in the K17 gene promoter and increases the level of transcription. The site is a context-independent GAS, and its sequence is highly homologous to those of GASs characterized in other IFN-γ-regulated genes. These results mean that the lymphocytes, via the IFN-γ signaling pathway, can induce not only the expression of cell surface proteins but also the expression of cytoplasmic cytoskeletal protein.

The presence of keratin K17 in vivo in psoriatic plaques and its absence in healthy interfollicular epidermis have been known. Results in Fig. 1 show that K17 can be induced experimentally in vivo by causing a DTH allergic reaction. We believe that K17 is induced whenever a sufficient amount of IFN-γ is present in the epidermis. In addition to psoriasis, these circumstances could include lichen planus, allergic contact dermatitis, atopic dermatitis, lupus erythematosus, and possibly tuberculoid leprosy and cutaneous T-cell lymphomas.

Whereas K17 is inducible by IFN-γ in the epidermis, it is present constitutively in a variety of other healthy, normal epithelia, in those epithelial cells that change shape frequently and exert force on the extracellular matrix and on other cells. It is possible that a specific function of K17, not shared by other keratins, is to participate in the contractile apparatus. K17 is induced by IFN-γ at a later stage of wound healing, when the lymphocytes are already present. At this point, the wound may be reepithelialized and the provisional extracellular matrix and basement membrane components may be laid down. The nascent extracellular structures may need physical reorganization. Dermal fibroblasts can contract and restructure a collagen-based extracellular matrix (2). The presence of keratin K17 could allow keratinocytes to participate, along with dermal fibroblasts, in rearranging the extracellular matrix in the later stages of would healing.

ACKNOWLEDGMENTS

Our special thanks go to M. Simon for the generous supply of keratinocytes. We thank J. Darnell, C. Basilico, D. Levi, and J. Vilček for comments on the manuscript. We also thank J. Filipovska for cloning the K17 functional gene, E. Collado Nuñez for the synthetic oligonucleotides, and J. Avins for secretarial help. We appreciate the gifts of antibody AE3 from T.-T. Sun, ICAM-1 clone from S. W. Caughman, and K8 and K18 clones from R. G. Oshima.

Our research was supported by National Institutes of Health grants AR30682 and AR39176 and NYU Skin Disease Research Center grant AR39749. M.B. is a recipient of an Irma T. Hirschl Career Scientist Award.

C.-K.J. and S.F. contributed equally to this work.

REFERENCES

1. **Barker, J. N. W. N., M. H. Allen, and D. M. MacDonald.** 1989. The effect of in vivo interferon-gamma on the distribution of LFA-1 and ICAM-1 in normal human skin. J. Invest. Dermatol. **93:**439–442.
2. **Bell, E., B. Ivarsson, and C. Merrill.** 1979. Production of a tissue-like structure by contraction of collagen lattices by human fibroblasts of different proliferative potential in vitro. Proc. Natl. Acad. Sci. USA **76:**1274–1278.
3. **Bernerd, F., T. Magnaldo, I. M. Freedberg, and M. Blumenberg.** 1993. Expression of the carcinoma-associated keratin K6 and the role of AP-1 proto-oncoproteins. Gene Expression **3:**187–199.
4. **Chang, E. H., J. Ridge, R. Black, Z.-Q. Zou, T. Masnyk, P. Noguchi, and J. B. Harford.** 1987. Interferon-γ induces altered oncogene expression and terminal differentiation in A431 cells. Proc. Soc. Exp. Biol. Med. **186:**319–326.
5. **Cooper, D., and T.-T. Sun.** 1986. Monoclonal antibody analysis of bovine epithelial keratins. J. Biol. Chem. **261:**4646–4654.
6. **Decker, T., D. J. Lew, and J. E. Darnell, Jr.** 1991. Two distinct alpha-interferon-dependent signal transduction pathways may contribute to activation of transcription of the guanylate-binding protein gene. Mol. Cell. Biol. **11:**5147–5153.
7. **Decker, T., D. J. Lew, J. Mirkovitch, and J. E. Darnell, Jr.** 1991. Cytoplasmic activation of GAF, an IFN-γ-regulated DNA-binding factor. EMBO J. **10:**927–932.
8. **Degitz, K., L. Lian-Jie, and S. W. Caughman.** 1991. Cloning and characterization of the 5′-transcriptional regulatory region of the human intercellular adhesion molecule 1 gene. J. Biol. Chem. **266:**14024–14030.
9. **de Jong, E. M. G. J., I. M. M. J. van Vlijmen, P. E. J. van Erp, F. C. S. Ramaekers, S. M. Troyanovski, and P. C. M. van de Kerhof.** 1991. Keratin 17: a useful marker in anti-psoriatic therapies. Arch. Dermatol. Res. **283:**480–482.
10. **Dustin, M. L., K. H. Singer, D. T. Tuck, and T. A. Springer.** 1988. Adhesion of T lymphoblasts to epidermal keratinocytes is regulated by intercellular adhesion molecule 1 (ICAM-1). J. Exp. Med. **167:**1323–1340.
11. **Ebner, R., and R. Derynck.** 1991. Epidermal growth factor and transforming growth factor-α: differential intracellular routing and processing of ligand-receptor complexes. Cell. Regul. **2:**599–612.
12. **Eron, L. J., F. Judson, S. Tucker, et al.** 1986. Interferon therapy for condylomata acuminata. N. Engl. J. Med. **315:**1059–1064.
13. **Flohr, T., U. Buwitt, B. Bonnekoh, T. Decker, and E. C. Böttger.** 1992. Interferon-γ regulates expression of a novel keratin class I

gene. Eur. J. Immunol. **22**:975–979.

14. **Fu, X.-Y., C. Schindler, T. Improta, R. Aebersold, and J. E. Darnell, Jr.** 1992. The proteins of ISGF-3, the interferon α-induced transcriptional activator, define a gene family involved in signal transduction. Proc. Natl. Acad. Sci. USA **89**:7840–7843.
15. **Fu, X.-Y., and J.-J. Zhang.** 1993. Transcription factor p91 interacts with the epidermal growth factor receptor and mediates activation of the c-*fos* gene promoter. Cell **74**:1135–1145.
16. **Gutch, M. J., C. Daly, and N. C. Reich.** 1992. Tyrosine phosphorylation is required for activation of an α interferon-stimulated transcription factor. Proc. Natl. Acad. Sci. USA **89**:11411–11415.
17. **Imam, A. M. A., A. M. Ackrill, T. C. Dale, I. M. Kerr, and G. R. Stark.** 1990. Transcription factors induced by interferons α and γ. Nucleic Acids Res. **18**:6573–6580.
18. **Jiang, C.-K., H. S. Epstein, M. Tomic, I. M. Freedberg, and M. Blumenberg.** 1990. Epithelial-specific keratin gene expression: identification of a 300 base-pair controlling segment. Nucleic Acids Res. **18**:247–253.
19. **Jiang, C.-K., H. S. Epstein, M. Tomic, I. M. Freedberg, and M. Blumenberg.** 1991. Functional comparison of the upstream regulatory DNA sequences of four human epidermal keratin genes. J. Invest. Dermatol. **96**:162–167.
20. **Jiang, C. K., T. Magnaldo, M. Ohtsuki, I. M. Freedberg, F. Bernerd, and M. Blumenberg.** 1993. EGF and TGFα specifically induce the activation- and hyperproliferation-associated keratins K#6 and K#16. Proc. Natl. Acad. Sci. USA **90**:6786–6790.
21. **Kaplan, G., M. D. Witmer, I. Nath, R. M. Steinman, S. Laal, H. K. Prasad, E. N. Sarno, U. Elvers, and Z. A. Cohn.** 1986. Influence of delayed immune reactions on human epidermal keratinocytes. Proc. Natl. Acad. Sci. USA **83**:3469–3473.
22. **Khan, K. D., K. Shuai, G. Lindwall, S. E. Maher, J. E. Darnell, Jr., and A. L. M. Bothwell.** 1993. Induction of the Ly-6A/E gene by both interferon α/β and γ requires a DNA element to which a tyrosine phosphorylated 91 kDa protein binds. Proc. Natl. Acad. Sci. USA **90**:6806–6811.
23. **Krueger, J. G., J. F. Krane, M. Carter, and A. B. Gottlieb.** 1990. Role of growth factors, cytokines, and their receptors in the pathogenesis of psoriasis. J. Invest. Dermatol. **94**:135S–140S.
24. **Kupper, T. S.** 1990. Role of epidermal cytokines, p. 285–305. *In* J. J. Oppenheim and E. M. Shevach (ed.), Immuno-physiology. The role of cells and cytokines in immunity and inflammation. Oxford University Press, New York.
25. **Levy, D., and J. E. Darnell, Jr.** 1990. Interferon-dependent transcriptional activation: signal transduction without second messenger involvement? New Biol. **2**:923–928.
26. **Mirkovitch, J., T. Decker, and J. E. Darnell, Jr.** 1992. Interferon induction of gene transcription analyzed by in vivo footprinting. Mol. Cell. Biol. **12**:1–9.
27. **Nickoloff, B. J., C. E. M. Griffiths, and J. N. W. N. Barker.** 1990. The role of adhesion molecules, chemotactic factors, and cytokines in inflammatory and neoplastic skin disease—1990 update. J. Invest. Dermatol. **94**:151S–157S.
28. **O'Guin, W. M., A. Schermer, and T.-T. Sun.** 1985. Immunofluorescence staining of keratin filaments in cultured epithelial cells. J. Tissue Culture Methods **9**:12–128.
29. **Ohtsuki, T., S. Flanagan, I. M. Freedberg, and M. Blumenberg.** 1993. A cluster of five nuclear proteins regulates keratin gene transcription. Gene Expression **3**:201–213.
30. **Pittelkow, M. R., R. J. Coffey, Jr., and H. L. Moses.** 1988. Keratinocytes produce and are regulated by transforming growth factors, p. 211–224. *In* L. M. Milstone and R. L. Edelson (ed.), Endocrine, metabolic and immunologic functions of keratinocytes, vol. 548. New York Academy of Sciences, New York.
31. **Reid, L. E., A. H. Brasnett, C. S. Gilbert, A. C. G. Porter, D. R. Gewert, G. R. Stark, and I. M. Kerr.** 1989. A single DNA response element can confer inducibility by both α- and γ-interferons. Proc. Natl. Acad. Sci. USA **86**:840–844.
32. **Ruff-Jamison, S., K. Chen, and S. Cohen.** 1993. Induction by EGF and interferon-γ of tyrosine phosphorylated DNA binding proteins in mouse liver nuclei. Science **261**:1733–1736.
33. **Savtchenko, E. S., M. Tomic, R. Ivker, and M. Blumenberg.** 1990. Three parallel linkage groups of human acidic keratin genes. Genomics **7**:394–407.
34. **Schindler, C., X.-Y. Fu, T. Improta, R. Aebersold, and J. E. Darnell, Jr.** 1992. Proteins of transcription factor ISGF-3: one gene encodes the 91- and 84-kDa ISGF-3 proteins that are activated by interferon α. Proc. Natl. Acad. Sci. USA **89**:7836–7839.
35. **Sen, G. C., and P. Lengyel.** 1992. The interferon system. A bird's eye view of its biochemistry. J. Biol. Chem. **267**:5017–5020.
36. **Shuai, K., C. Schindler, V. R. Prezioso, and J. E. Darnell, Jr.** 1992. Activation of transcription by IFN-γ: tyrosine phosphorylation of a 91-kD DNA binding protein. Science **258**:1808–1812.
37. **Shuai, K., A. Ziemiecki, A. F. Wilks, A. G. Harpur, H. B. Sadowski, M. Z. Gilman, and J. E. Darnell.** 1993. Polypeptide signalling to the nucleus through tyrosine and phosphorylation of Jak and Stat proteins. Nature (London) **366**:580–585.
38. **Steinert, P. M., and D. R. Roop.** 1988. Molecular and cellular biology of intermediate filaments. Annu. Rev. Biochem. **57**:593–625.
39. **Troyanovski, S. M., R. E. Leube, and W. W. Franke.** 1992. Characterization of the human gene encoding cytokeratin 17 and its expression pattern. Eur. J. Cell Biol. **59**:127–137.
40. **Velazquez, L., M. Fellous, G. R. Star, and S. Pellegrini.** 1992. A protein tryosine kinase in the interferon α/β signaling pathway. Cell **70**:313–322.
41. **Vilček, J.** 1990. Interferons, p. 3–38. *In* M. B. Sporn and A. B. Roberts (ed.), Peptide growth factors and their receptors II. Springer-Verlag, New York.
42. **Wilson, K. C., and D. S. Feinbloom.** 1992. Interferon γ rapidly induces in human monocytes a DNA-binding factor that recognizes the γ response region within the promoter of the gene for the high-affinity Fcγ receptor. Proc. Natl. Acad. Sci. USA **89**:11964–11968.