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Author(s):
Beer, Hans-Dietmar; Munding, Christine; Dubois, Nicole; Mamie, Céline; Hohl, Daniel; Werner, Sabine

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The Estrogen-responsive B Box Protein

A NOVEL REGULATOR OF KERATINOCYTE DIFFERENTIATION*

Hans-Dietmar Beer‡§, Christine Munding‡, Nicole Dubois‡, Céline Mamie‡, Daniel Hohl¶, and Sabine Werner‡

From the ‡Institute of Cell Biology, Department of Biology, ETH Zürich, CH-8093 Zürich, Switzerland and the ¶Service de Dermatologie, CHUV Hôpital Beaumont, BT-437 CH-1011 Lausanne, Switzerland

Keratinocyte growth factor (KGF) regulates proliferation, differentiation, migration, and survival of different types of epithelial cells, including keratinocytes of the skin. To gain insight into the mechanisms underlying these multiple functions, we searched for KGF-regulated genes in keratinocytes. Using the differential display reverse transcriptase-PCR technology, we identified the gene encoding the estrogen-responsive B box protein (EBBP) which has as yet not been functionally characterized. The full-length murine and human EBBP cDNAs were cloned and fully sequenced. They were shown to encode 75-kDa proteins, which are mainly localized in the cytoplasm of keratinocytes *in vitro* and *in vivo*. *In vivo*, EBBP was found at high levels in the KGF- and epidermal growth factor-responsive basal keratinocytes of human skin, but the expression was downregulated in the hyperthickened epithelium of skin wounds. Stable overexpression of EBBP in HaCaT keratinocytes did not affect the proliferation rate of the transfected cells, but enhanced the early differentiation process. These results suggest that the presence of EBBP in basal keratinocytes is important for the differentiation capacity of these cells, and that downregulation of EBBP expression in a hyperproliferative epithelium is required to maintain the cells in a non-differentiated stage.

The epidermis forms the protective cover of the body surface. It is a multilayered renewal tissue composed mainly of keratinocytes. Terminally differentiated and dead cells are continuously lost from its surface and replaced by cells originating from the basal layer. Under normal conditions only cells touching on the basement membrane are able to proliferate (1). Transient hyperproliferation of keratinocytes occurs at the wound margins after skin injury (2), and permanent, uncontrolled hyperproliferation, together with abnormal differentiation of keratinocytes is a characteristic feature of major human skin diseases, such as psoriasis and epidermal cancers (3, 4). Therefore, the balance between proliferation and differentia-

tion must be tightly regulated. Cell-cell and cell-matrix interactions, as well as soluble factors such as hormones and growth factors are involved in this process, but the intracellular effectors have only partially been identified.

To identify novel regulators of keratinocyte proliferation and differentiation, we searched for genes that are regulated by keratinocyte growth factor (KGF).[1] KGF (fibroblast growth factor 7, FGF-7) is a member of the FGF family, which is predominantly expressed by mesenchymal cells, but not by epithelial cells (5). It acts in a paracrine manner by binding to its receptor, a splice variant of FGF receptor 2 that is expressed by different types of epithelial cells, including keratinocytes of the skin (6). This ligand-receptor interaction has been implicated in morphogenetic processes of epithelial tissues and in cell survival under stress conditions (7). KGF expression is strongly induced upon injury to various epithelial tissues in normal and pathological situations (7, 8, 9), and activation of the KGF receptor was shown to be important for efficient re-epithelialization of skin wounds (10).

In vitro, KGF stimulates keratinocyte migration and proliferation (11, 12), and it inhibits terminal differentiation and apoptosis of these cells (13). Nevertheless, it also induces the expression of differentiation–specific proteins in response to an increase in the extracellular Ca^{2+} concentration, and it has been suggested that KGF plays a crucial role in the initiation of the early differentiation program (14, 15).

To gain insight into the changes in gene expression after binding of KGF to its receptor, we performed differential display RT-PCR (DDRT-PCR) with RNAs from quiescent and KGF-stimulated HaCaT keratinocytes. By this method we identified the *ebbp* gene as a target of KGF action. The latter was originally identified as a gene, which is regulated by estrogen and tamoxifen in human mammary epithelial cells stably expressing an estrogen receptor mutant (16). It is ubiquitously expressed in human tissues, but the function of the encoded protein is completely unknown. In the present study, we have characterized the EBBP protein, and we demonstrate a novel role of EBBP in the regulation of keratinocyte differentiation.

EXPERIMENTAL PROCEDURES

Growth Factor Treatment of HaCaT Keratinocytes—HaCaT keratinocytes (17) were grown to confluence in Dulbecco's modified Eagle's medium (Sigma, Munich, Germany) containing 1% penicillin/streptomycin and 10% fetal calf serum (FCS; Amimed, Allschwil, Switzerland). They were rendered quiescent by serum starvation for 16 to 24 h, and subsequently treated with fresh Dulbecco's modified Eagle's medium

[1] The abbreviations used are: KGF, keratinocyte growth factor; FGF, fibroblast growth factor; DDRT-PCR, differential display RT-PCR; FCS, fetal calf serum; EGF, epidermal growth factor; HA, hemagglutinin; RBCC, RING finger-B box-coiled coil; TRIM, tripartite motif.

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containing 10 ng/ml KGF, 10 ng/ml fibroblast growth factor 10 (FGF-10), 20 ng/ml epidermal growth factor (EGF), 1 ng/ml transforming growth factor-β1, 100 units/ml interleukin-1β, 300 units/ml tumor necrosis factor-α, or 10% fetal calf serum (FCS), respectively. Cells were harvested at different time points after growth factor or serum stimulation and used for RNA isolation or for preparation of protein lysates. Growth factors and cytokines were purchased from Roche Molecular Biochemicals (Mannheim, Germany) or R&D Systems (Abingdon, UK).

Transfection of HaCaT Cells and COS-1 Cells—For transient and stable transfection the LipofectAMINE 2000 Reagent was used according to the manufacturer's instructions (Invitrogen, Basel, Switzerland). For the generation of stable cell lines HaCaT cells were co-transfected with an expression plasmid that contains the EBBP cDNA under the control of the cytomegalovirus promoter together with a plasmid that confers resistance to geneticin (G418). After removal of the transfection mixture cells were trypsinized, re-seeded at lower cell density, and incubated in medium containing 400 mg/liter G418 (Invitrogen) until most of the cells had died and resistant colonies had appeared. The latter were isolated, expanded, and analyzed for the expression of EBBP by Western blotting. Transfected cells were cultured in G418-containing medium for at least 10 passages until used for experiments.

Cell Lysis and Immunoblotting—Preparation of total cell lysates was performed as described previously (18), and protein concentrations were determined using the BCA kit (Pierce, Rockford, IL). Isolation of cytoplasmic and nuclear proteins was performed by hypotonic lysis followed by high salt extraction of nuclei (19). Proteins were separated by SDS-polyacrylamide gel electrophoresis (8–12%) and transferred to nitrocellulose membranes. Membranes were incubated with the primary antibodies followed by alkaline phosphatase- or horseradish peroxidase-conjugated secondary antibodies. Antibody-binding proteins were detected with the nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (Promega, Madison, WI) or enhanced chemoluminescence (ECL) (Amersham Biosciences) detection systems, respectively. The following antibodies were used: mouse monoclonal antibodies directed against keratin 10 (Dako, Glostrup, Denmark), involucrin (NeoMarkers, Fremont, CA), p53 (Oncogene Research Products, Cambridge, MA), β-actin (Sigma), or the influenza virus hemagglutinin epitope (HA; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), a goat polyclonal antibody against RB (Santa Cruz) and rabbit polyclonal antibodies directed against keratin 6 (Babco, Richmond, CA), and against the peptide CKGIDRKGEERNS, corresponding to amino acids 446–458 (Fig. 2*B*) EBBP. This peptide is fully conserved in the murine protein. The EBBP antibody was generated and affinity purified by Eurogentec Bel S.A. (Seraing, Belgium). All secondary antibodies for Western blots were from Promega.

Immunofluorescence—Indirect immunofluorescence was performed with acetone-fixed cells or frozen sections from human skin or with ethanol/acetic acid (95/1%) fixed paraffin sections of normal and wounded mouse skin. Cells or tissue sections were incubated with primary antibodies diluted in 1% bovine serum albumin, followed by the secondary antibody diluted in 12% bovine serum albumin. The latter were either anti-mouse IgG-fluorescein isothiocyanate (Sigma) or anti-rabbit IgG-Cy3™ (Jackson ImmunoResearch, West Grove, PA).

RNA Isolation and RNase Protection Assay—Isolation of total cellular RNA and RNase protection assays were performed as described (7, 20). The following cDNA fragments were used as templates: a fragment corresponding to nucleotides 361–624 of the coding region of the human EBBP cDNA (GenBank™ AF096870), a fragment corresponding to part of the coding region of the murine EBBP cDNA (nucleotides 84–377), a 120-bp fragment corresponding to nucleotides 566–685 of the mouse glyceraldehyde-3-phosphate dehydrogenase cDNA (GenBank™ M32599), and a 116-bp fragment corresponding to nucleotides 580–695 of the human glyceraldehyde-3-phosphate dehydrogenase cDNA (GenBank™ BC 001601).

DDRT-PCR—DDRT-PCR was carried out as previously described (21). The radioactive band was isolated from the gel, eluted in water, and after dilution, reamplified and cloned.

Cloning of Full-length EBBP cDNAs—The DDRT-PCR cDNA fragment was used to screen a cDNA library from KGF-stimulated HaCaT keratinocytes (21). 19 positive plaques were recovered from the bacterial dishes, replated, and used for a further round of hybridization. Positive phage plaques were used for an *in vivo* excision reaction of the pBluescript SK(−) phagemid from the Uni-ZAP vector (according to Stratagene). The rescued phagemids were then used to transform *Escherichia coli* SOLR and preparations of the plasmids were used for subsequent restriction analysis. The longest cDNAs were fully se-
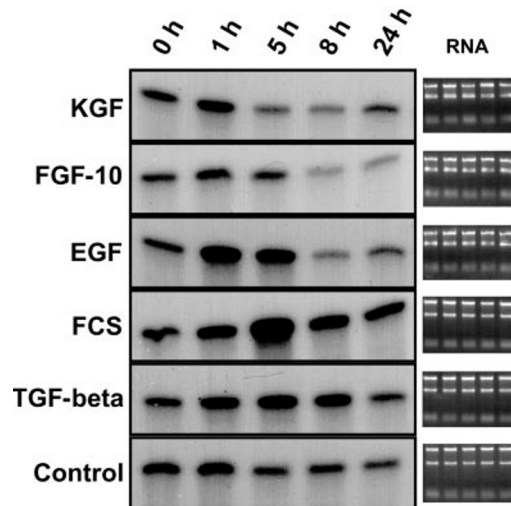

FIG. 1. **Regulation of *ebbp* expression by KGF, FGF-10, EGF, transforming growth factor-β1, and serum.** HaCaT keratinocytes were rendered quiescent by serum starvation and stimulated with FCS or with different purified growth factors as indicated. Samples of 20 μg of total cellular RNA were analyzed by RNase protection assay using a ^{32}P-labeled antisense probe corresponding to part of the coding region of the EBBP cDNA. The time after growth factor or serum addition is indicated on the *top*. As a loading control, 1 μg of the RNA samples was loaded on a 1% agarose gel and stained with ethidium bromide.

quenced in both orientations. For the cloning of the murine cDNA a probe from the coding region of human EBBP was used to screen a mouse skin wound cDNA library (22).

Human Skin Biopsies—Normal human skin was obtained from skin transplants of patients attending the Dermatology Department of the University of Cologne for surgery (*n* = 6, aged 31–79 years). All samples included the dermis and the epidermis. The biopsies were immediately frozen in tissue freezing medium. All patients signed informed consent for the Department of Dermatology, University of Cologne, approved by the Institutional Commission of Ethics (Az. 9645/96).

Wounding and Preparation of Wound Tissue—Two independent wound-healing experiments were performed. For each experiment, 24 BALB/c mice (8–12 weeks of age) were anaesthetized with a single intraperitoneal injection of ketamine/xylazine. The hair on the back of the animal was shaved, and the skin was wiped with 70% ethanol. Four full-thickness excisional wounds (4 mm diameter, 3–4 mm apart) were generated on the back of each animal by excising skin and *panniculus carnosus*. The excised skin served as a control. The wounds were allowed to dry to form a scab. At different time points after injury (1–14 days), animals were sacrificed, and the complete wounds including 2 mm of the margins were isolated. At each time point, the tissue from 4 animals was combined, immediately frozen in liquid nitrogen, and used for RNA isolation or for preparation of protein lysates. For immunohistochemistry, wounds were isolated as described above, bisected, fixed in 95% ethanol, 1% acetic acid, embedded in paraffin, and sectioned.

RESULTS

The ebbp Gene Is a Novel Target of KGF Action in Keratinocytes—To identify novel KGF-regulated genes in keratinocytes, we used the immortalized, but non-transformed HaCaT cell line, which has been shown to respond to this growth factor (21). These cells were grown to confluence, serum-starved overnight, and subsequently stimulated with KGF. Total cellular RNA was isolated 5 and 8 h after KGF addition, and used for DDRT-PCR (21). A fragment was amplified predominantly from the control cDNA and to a much lesser extent from cDNAs of KGF-stimulated cells. This fragment was isolated from the gel, re-amplified by PCR, and cloned. Sequencing revealed that the fragment corresponds to part of the 3′ end of the untranslated region of the gene encoding EBBP (16), which is also called TRIM16 (23).

To confirm the KGF-regulated expression of *ebbp* we performed RNase protection assays using a template corresponding
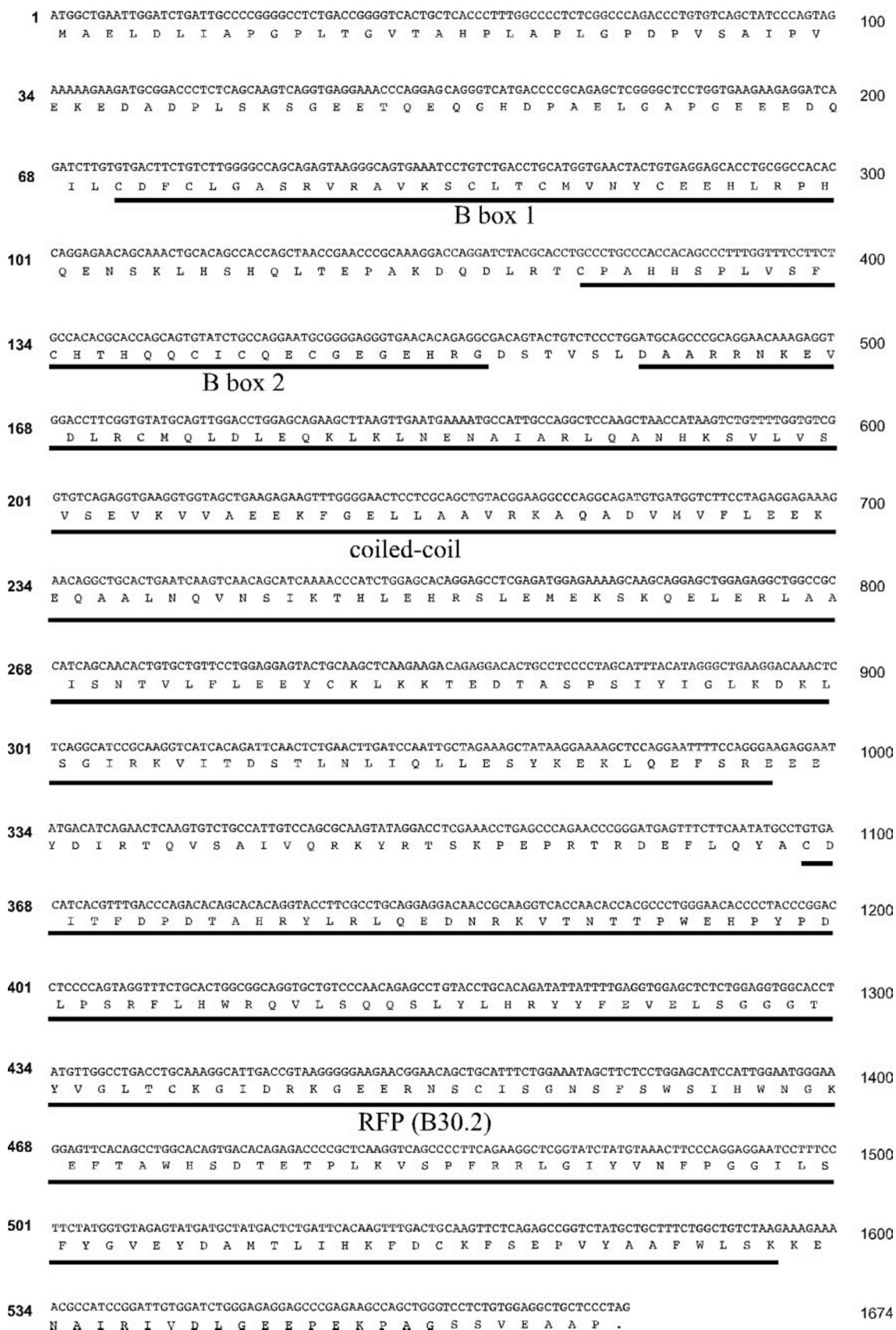
EBBP and Keratinocyte Differentiation

A

```
1    ATGGCTGAATTGGATCTGATTGCCCCGGGGCCTCTGACCGGGGTCACTGCTCACCCTTTGGCCCCTCTCGGCCCAGACCCTGTGTCAGCTATCCCAGTAG    100
     M  A  E  L  D  L  I  A  P  G  P  L  T  G  V  T  A  H  P  L  A  P  L  G  P  D  P  V  S  A  I  P  V

34   AAAAGAAGATGCGGACCCTCTCAGCAAGTCAGGTGAGGAAACCCAGGAGCAGGGTCATGACCCCGCAGAGCTCGGGGCTCCTGGTGAAGAAGAGGATCA    200
     E  K  E  D  A  D  P  L  S  K  S  G  E  E  T  Q  E  Q  G  H  D  P  A  E  L  G  A  P  G  E  E  D  Q

68   GATCTTGTGTGACTTCTGTCTTGGGGCCAGCAGAGTAAGGGCAGTGAAATCCTGTCTGACCTGCATGGTGAACTACTGTGAGGAGCACCTGCGGCCACAC    300
     I  L  C  D  F  C  L  G  A  S  R  V  R  A  V  K  S  C  L  T  C  M  V  N  Y  C  E  E  H  L  R  P  H
            ‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾
                                          B box 1

101  CAGGAGAACAGCAAACTGCACAGCCACCAGCTAACCGAACCCGCAAAGGACCAGGATCTACGCACCTGCCCTGCCCACCACAGCCCTTTGGTTTCCTTCT    400
     Q  E  N  S  K  L  H  S  H  Q  L  T  E  P  A  K  D  Q  D  L  R  T  C  P  A  H  H  S  P  L  V  S  F
                                                         ‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾

134  GCCACACGCACCAGCAGTGTATCTGCCAGGAATGCGGGGAGGGTGAACACAGAGGCGACAGTACTGTCTCCCTGGATGCAGCCCGCAGGAACAAAGAGGT    500
     C  H  T  H  Q  Q  C  I  C  Q  E  C  G  E  G  E  H  R  G  D  S  T  V  S  L  D  A  A  R  R  N  K  E  V
     ‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾             ‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾
                        B box 2

168  GGACCTTCGGTGTATGCAGTTGGACCTGGAGCAGAAGCTTAAGTTGAATGAAAATGCCATTGCCAGGCTCCAAGCTAACCATAAGTCTGTTTTGGTGTCG    600
     D  L  R  C  M  Q  L  D  L  E  Q  K  L  K  L  N  E  N  A  I  A  R  L  Q  A  N  H  K  S  V  L  V  S
     ‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾

201  GTGTCAGAGGTGAAGGTGGTAGCTGAAGAGAAGTTTGGGGAACTCCTCGCAGCTGTACGGAAGGCCCAGGCAGATGTGATGGTCTTCCTAGAGGAGAAG    700
     V  S  E  V  K  V  V  A  E  E  K  F  G  E  L  L  A  A  V  R  K  A  Q  A  D  V  M  V  F  L  E  E  K
     ‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾
                                          coiled-coil

234  AACAGGCTGCACTGAATCAAGTCAACAGCATCAAAACCCATCTGGAGCACAGGAGCCTCGAGATGGAGAAAAGCAAGCAGGAGCTGGAGAGGCTGGCCGC    800
     E  Q  A  A  L  N  Q  V  N  S  I  K  T  H  L  E  H  R  S  L  E  M  E  K  S  K  Q  E  L  E  R  L  A  A
     ‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾

268  CATCAGCAACACTGTGCTGTTCCTGGAGGAGTACTGCAAGCTCAAGAAGACAGAGGACACTGCCTCCCCTAGCATTTACATAGGGCTGAAGGACAAACTC    900
     I  S  N  T  V  L  F  L  E  E  Y  C  K  L  K  K  T  E  D  T  A  S  P  S  I  Y  I  G  L  K  D  K  L
     ‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾

301  TCAGGCATCCGCAAGGTCATCACAGATTCAACTCTGAACTTGATCCAATTGCTAGAAAGCTATAAGGAAAAGCTCCAGGAATTTTCCAGGGAAGAGGAAT    1000
     S  G  I  R  K  V  I  T  D  S  T  L  N  L  I  Q  L  L  E  S  Y  K  E  K  L  Q  E  F  S  R  E  E  E
     ‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾

334  ATGACATCAGAACTCAAGTGTCTGCCATTGTCCAGCGCAAGTATAGGACCTCGAAACCTGAGCCCAGAACCCGGGATGAGTTTCTTCAATATGCCTGTGA    1100
     Y  D  I  R  T  Q  V  S  A  I  V  Q  R  K  Y  R  T  S  K  P  E  P  R  T  R  D  E  F  L  Q  Y  A  C  D
                                                                                              ‾‾‾‾‾

368  CATCACGTTTGACCCAGACACAGCACACAGGTACCTTCGCCTGCAGGAGGACAACCGCAAGGTCACCAACACCACGCCCTGGGAACACCCTACCCGGAC    1200
     I  T  F  D  P  D  T  A  H  R  Y  L  R  L  Q  E  D  N  R  K  V  T  N  T  T  P  W  E  H  P  Y  P  D
     ‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾

401  CTCCCCAGTAGGTTTCTGCACTGGCGGCAGGTGCTGTCCCAACAGAGCCTGTACCTGCACAGATATTATTTTGAGGTGGAGCTCTCTGGAGGTGGCACCT    1300
     L  P  S  R  F  L  H  W  R  Q  V  L  S  Q  Q  S  L  Y  L  H  R  Y  Y  F  E  V  E  L  S  G  G  G  T
     ‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾

434  ATGTTGGCCTGACCTGCAAAGGCATTGACCGTAAGGGGGAAGAACGGAACAGCTGCATTTCTGGAAATAGCTTCTCCTGGAGCATCCATTGGAATGGGAA    1400
     Y  V  G  L  T  C  K  G  I  D  R  K  G  E  E  R  N  S  C  I  S  G  N  S  F  S  W  S  I  H  W  N  G  K
     ‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾
                                          RFP (B30.2)

468  GGAGTTCACAGCCTGGCACAGTGACACAGAGACCCCGCTCAAGGTCAGCCCCTTCAGAAGGCTCGGTATCTATGTAAACTTCCCAGGAGGAATCCTTTCC    1500
     E  F  T  A  W  H  S  D  T  E  T  P  L  K  V  S  P  F  R  R  L  G  I  Y  V  N  F  P  G  G  I  L  S
     ‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾

501  TTCTATGGTGTAGAGTATGATGCTATGACTCTGATTCACAAGTTTGACTGCAAGTTCTCAGAGCCGGTCTATGCTGCTTTCTGGCTGTCTAAGAAAGAAA    1600
     F  Y  G  V  E  Y  D  A  M  T  L  I  H  K  F  D  C  K  F  S  E  P  V  Y  A  A  F  W  L  S  K  K  E
     ‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾

534  ACGCCATCCGGATTGTGGATCTGGGAGAGGAGCCCGAGAAGCCAGCTGGGTCCTCTGTGGAGGCTGCTCCCTAG    1674
     N  A  I  R  I  V  D  L  G  E  E  P  E  K  P  A  G  S  S  V  E  A  A  P  .
```

FIG. 2. **Nucleotide and deduced amino acid sequence of murine EBBP.** *A*, *numbers* on the *left-hand* side refer to the amino acid position and those on the *right* to the nucleotide position (GenBank™ accession number AF449496). The B boxes, coiled-coil, and B30.2 domains are *underlined*. *B*, alignment of the human (*upper sequence*) and mouse (*lower sequence*) EBBP amino acid sequence. Amino acids, which are identical in the human and murine proteins are indicated by *asterisks*.

B

```
           ** **** *****     ** * *** **          ** *** **       *
1   MAELDLMAPGPLPRATAQPPAPLSPDSGSPSPDSGSASPVEEEDVGSSEK
    MAELDLIAPGPLTGVTAHPLAPLGPDP-------VSAIPVEKEDADPLSK

       *  ** **    * ** * *    *     ******  * * ************
50  LGRETEEQDSDSAEQGDPAGEGKEVL┌CDFCLDDTRRVKAVKSCLTCMVNY┐
    SGEETQEQGHDPAELGAPGEEEDQII│CDFCLGASR-VRAVKSCLTCMVNY│  B box1
                               └                       ┘
       ***** ***  * ** ** **** **     * ********   **     *****
100 ┌CEEHLQPH┐QVNIKLQSHLLTEPVKDHNWRY┌CPAHHSPLSAFCCPDQQCIC┐
    │CEEHLRPH│QENSKLHSHQLTEPAKDQDLRT│CPAHHSPLVSFCHTHQQCIC│  B box2
     └       ┘                      └                   ┘
       *  *     ** *     ********  **    * *  *****  ********  ****
150 ┌QDCCQ-EHSG┐HTIVSL┌DAARRDKEAELQCTQLDLERKLKLNENAISRLQA┐
    │QECGEGEHRG│DSTVSL│DAARRNKEVDLRCMQLDLEQKLKLNENAIARLQA│
     └         ┘      │                                  │
       *  ********** **  ***  ************* **  **************
200 │NQKSVLVSVSEVKAVAEMQFGELLAAVRKAQANVMPFLEEKEQAALNQAN│
    │NHKSVLVSVSEVKVVAEEKFGELLAAVRKAQADVMVFLEEKEQAALNQVN│  coiled
    │                                                  │  -coil
       ** *** **  ***********  ******* *******  *  ***    **
250 │GIKAHLEYRSAEMEKSKQELERMAAISNTVQFLEEYCKFKNTEDITFPSV│
    │SIKTHLEHRSLEMEKSKQELERLAAISNTVLFLEEYCKLKKTEDTASPSI│
    │                                                  │
       *  *************  **    ******  **  *****************
300 │YVGLKDKLSGIRKVITESTVHLIQLLENYKKKLQEFSKE┐EEYDIRTQVSA
    │YIGLKDKLSGIRKVITDSTLNLIQLLESYKEKLQEFSRE│EEYDIRTQVSA
    └                                       ┘

       ****  ******  **    *****  *********  ******  ********
350 VVQRKYWTSKPEPSTREQFLQYA┌YDITFDPDTAHKYLRLQEENRKVTNTT┐
    IYQRKYRTSKPEPRTRDEFLQYA│CDITFDPDTAHRYLRLQEDNRKVTNTT│
                           │                           │
       *****************************************   *  ***********
400 ┌PWEHPYPDLPSRFLHWRQVLSQQSLYLHRYYFEVEIFGAGTYVGLTCKGI│
    │PWEHPYPDLPSRFLHWRQVLSQQSLYLHRYYFEVELSGGGTYVGLTCKGI│
    │                                                  │  RFP
       ************** ****  *********  **  *****    ****** *  (B30.2)
450 │DRKGEERNSCISGNNFSWSLQWNGKEFTAWYSDMETPLKAGPFRRLGVYI│
    │DRKGEERNSCISGNSFSWSIHWNGKEFTAWHSDTETPLKVSPFRRLGIYV│
    │                                                  │
       **************** *** * *  ******************  *******
500 │DFPGGILSFYGVEYDTMTLVHNFACKFSEPVYAAFWLSK┐KESAIRIVDLG
    │NFPGGILSFYGVEYDAMTLIHKFDCKFSEPVYAAFWLSK│KENAIRIVDLG
    └                                       ┘

       ******* * *   **
550 EEPEKPAPSLVVTAP
    EEPEKPAGSSVEAAP
```

FIG. 2—*continued*

to part of the coding region of the EBBP cDNA. We observed a biphasic regulation of *ebbp* expression after addition of KGF to quiescent HaCaT cells (Fig. 1). After a minor initial increase (1 h after KGF stimulation), EBBP mRNA levels declined strongly within 5–8 h. The down-regulation was partially reversible, since the mRNA levels increased again after 24 h. A similar regulation of *ebbp* expression was also seen with FGF-10, another member of the FGF family, which binds to the same receptor as KGF (24). In this case, however, the down-regulation occurred later compared with KGF. Treatment of HaCaT cells with EGF, another

important mitogen for keratinocytes (25) or with serum, caused a similar biphasic regulation of *ebbp* expression, although the initial induction was stronger compared with the induction seen with KGF and FGF-10. By contrast, transforming growth factor-β1 had only a minor effect on EBBP mRNA levels (Fig. 1) and interleukin-1β or tumor necrosis factor-α had no effect (not shown). Incubation of the cells in the absence of any growth factor or cytokine resulted in a slight down-regulation of *ebbp* expression. Thus, regulation of *ebbp* expression appears to be specific for keratinocyte mitogens.
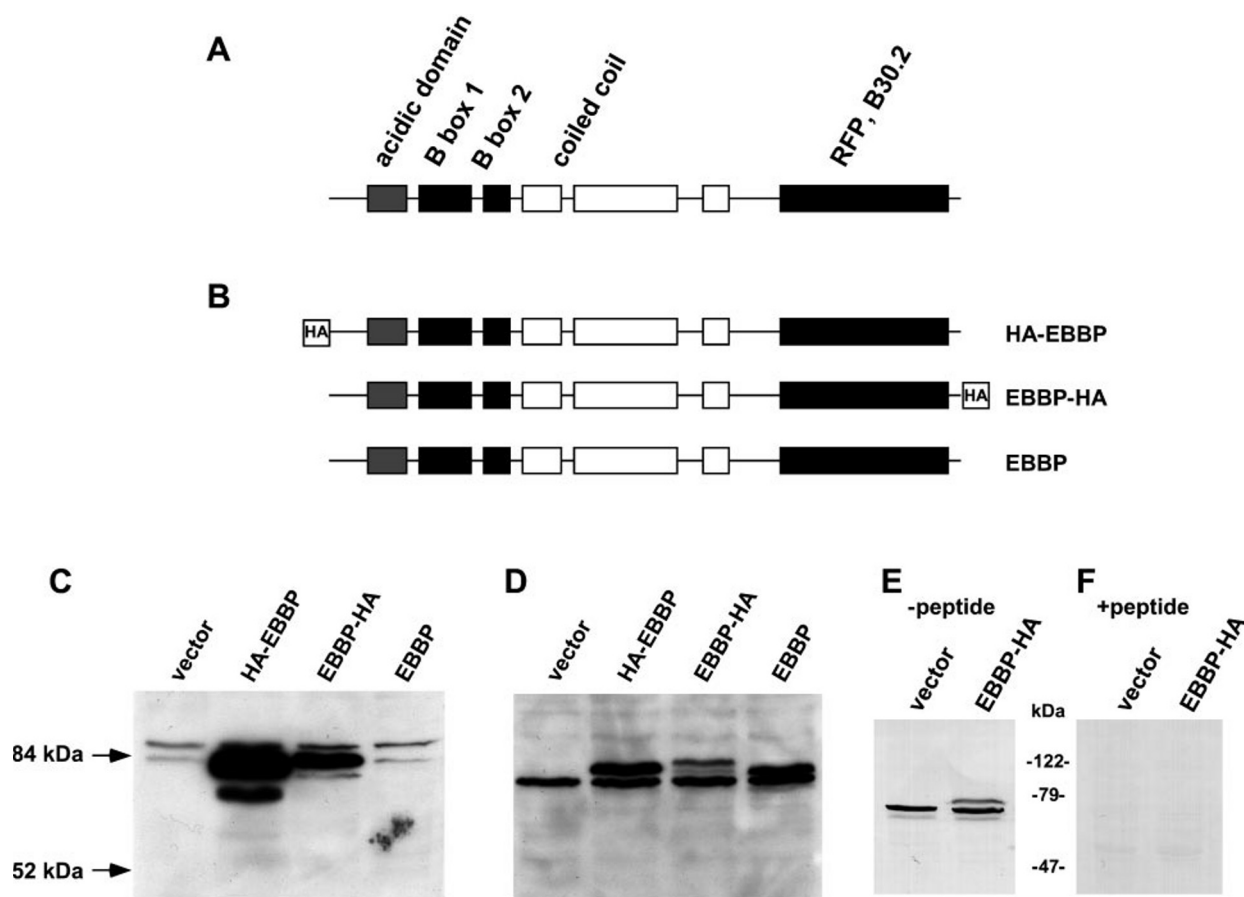
FIG. 3. **Structural domains and expression of the estrogen-responsive B box protein.** *A*, schematic representation of EBBP protein domains. Identification of protein domains was achieved with PIX (www.hgmp.mrc.ac.uk/) and BLAST (www.ncbi.nlm.nih.gov/BLAST/). *B*, schematic representation of EBBP proteins transiently expressed in COS-1 cells. A sequence coding for an HA epitope tag was cloned in-frame with EBBP at the amino or carboxyl terminus (HA-EBBP, EBBP-HA) or EBBP was expressed without tag (EBBP). *C* and *D*, COS-1 cells were transiently transfected with plasmids coding for HA-EBBP, EBBP-HA, and EBBP. Tagged proteins were detected with a monoclonal HA-antibody (*C*), and EBBP proteins with the polyclonal EBBP antibody (*D*). *E* and *F*, Western blot of total protein lysates of vector- and EBBP-HA-transfected HaCaT cells using the EBBP antibody before (*E*) and after (*F*) pretreatment with the immunization peptide.

Cloning of cDNAs Coding for Human and Murine EBBP—We subsequently isolated the full-length human and murine EBBP cDNAs from cDNA libraries of HaCaT cells or mouse skin wounds, respectively. Several positive clones were obtained, and the longest inserts were completely sequenced in both orientations. The cDNAs isolated from the HaCaT library correspond to the published sequence of the EBBP cDNA (Gen-Bank[TM] XM052478 and NM006470.2) and to six stretches on human chromosome 17 (GenBank[TM] AC005324), the known locus of the *ebbp* gene (17p11.2) (16). This finding suggests that the human *ebbp* gene includes 6 exons. The 3′ ends of the cDNAs isolated from the mouse skin wound library are identical to a partial cDNA of the tripartite motif (TRIM) protein 16 (GenBank[TM] AF220134.1), the putative murine homologue of human EBBP (23). The complete open reading frame of the murine *ebbp* gene is shown in Fig. 2*A*. The human EBBP protein consists of 564 amino acids and has an expected molecular weight of 64,000, whereas the mouse EBBP protein is only 558 amino acids in length (Fig. 2*B*).

The predicted EBBP proteins start at the amino terminus with a region containing a high percentage of negatively charged amino acids (Figs. 2, *A* and *B*, and 3*A*). This region is followed by two B boxes and three coiled-coil domains, which often mediate protein-protein interactions (26). The COOH terminus of EBBP is characterized by the presence of a B30.2 domain of as yet unknown function (27). The total sequence identity between the human and mouse EBBP proteins is 77%,

ranging from 41% in the acidic domain to 88% in the B30.2 domain. This domain composition is characteristic for members of the RING finger-B box-coiled coil (RBCC) protein family that play important roles in development, differentiation, and disease.

EBBP Is a 75-kDa Cytoplasmic Protein—To characterize the EBBP proteins we generated a polyclonal rabbit antiserum directed against a 13-amino acid peptide corresponding to the amino-terminal part of the B30.2 domain (amino acids 446–458 in Fig. 2*B*). This antibody should recognize both the human and the murine EBBP proteins, which are identical in this part of the B30.2 domain (Fig. 2*B*). To determine the specificity of the antibody, we fused the human EBBP cDNA in-frame with a sequence encoding an epitope tag of the influenza virus hemagglutinin (HA) (Fig. 3*B*). Full-length EBBP as well as proteins with the HA epitope at the amino terminus (HA-EBBP) or carboxyl terminus (EBBP-HA) were transiently expressed in COS-1 cells under the control of the cytomegalovirus promoter. The calculated molecular mass of the tagged proteins is 66 kDa. Using the HA antibody, proteins of an apparent molecular mass of about 78 kDa were detected in total cell lysates of EBBP-HA and HA-EBBP transfected cells (Fig. 3*C*). By contrast, these proteins were not detectable in vector-transfected cells or in cells transfected with the non-tagged proteins, indicating that they represent the tagged versions of EBBP. The affinity purified EBBP antibody recognized proteins of the same size, demonstrating a high specificity of this antiserum
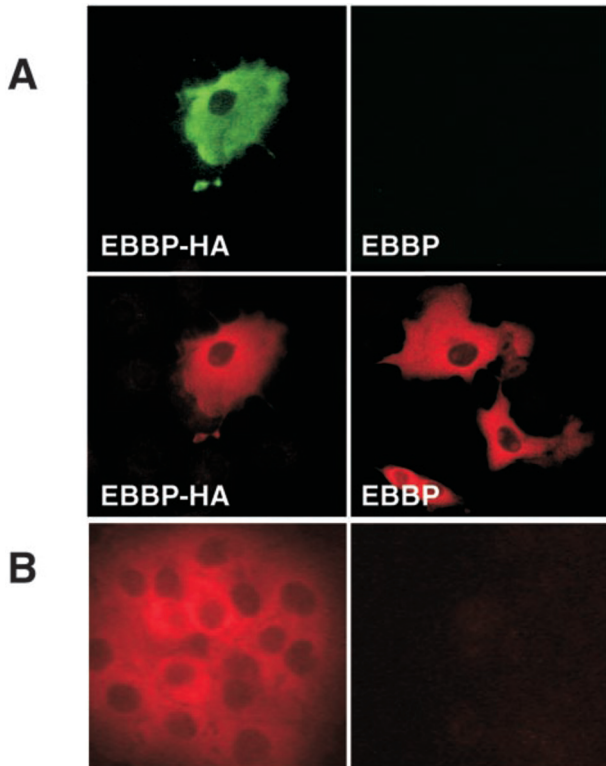
FIG. 4. **EBBP is predominantly localized in the cytoplasm of exponentially growing COS-1 cells and HaCaT cells.** *A*, COS-1 cells were transiently transfected with EBBP-HA and EBBP expression vectors as indicated and analyzed by indirect immunofluorescence with antibodies against the HA epitope (*upper panel*) or EBBP (*lower panel*). *B*, non-transfected HaCaT cells were analyzed by indirect immunofluorescence with the untreated EBBP antibody (*left panel*) or with the EBBP antibody that had been pretreated with the immunization peptide (*right panel*).

(Fig. 3*D*). In addition, it also detected the untagged transfected protein and a protein, which migrated slightly faster than the untagged EBBP (75 kDa). The latter is likely to represent endogenous EBBP, since it was also detected in vector-transfected cells (Fig. 3*D*) and non-transfected cells (not shown). The reason for the larger size of the untagged, recombinant EBBP compared with the endogenous protein is not known, but the presence of four additional amino acids at the amino terminus of the recombinant protein might at least partially explain this phenomenon. These amino acids result from the presence of a second ATG in the expression vector that is preceded by a KOZAK sequence for optimal translation. Alternatively, the difference in size may be due to different post-translational modification of the endogenous protein.

In non-transfected and vector-transfected HaCaT cells, the EBBP antibody also recognized a protein of about 75 kDa (Fig. 3*E*) which is likely to represent endogenous EBBP, and a slightly larger protein in HaCaT cells that were transiently transfected with the EBBP-HA cDNA. These proteins were not detected when the antibody was pre-incubated with an excess of the immunization peptide (Fig. 3*F*).

We subsequently performed immunofluorescence with the monoclonal HA and the polyclonal EBBP antibody. A strong cytoplasmic staining and a weak nuclear staining was observed with the HA antibody in EBBP-HA-transfected COS-1 cells (Fig. 4*A*, *upper panel*) but not in EBBP-transfected cells. A similar localization was observed with the EBBP antibody in vector-transfected, EBBP-HA- and EBBP transfected cells (Fig. 4*A*, *lower panel*, and data not shown). Most importantly, the distribution of EBBP was identical in COS-1 cells, which had

been transiently transfected with EBBP, EBBP-HA, or HA-EBBP expression vectors, demonstrating that the HA epitope tag does not affect the localization (data not shown). A similar staining pattern was observed with the EBBP antibody in HaCaT cells (Fig. 4*B, left panel*). The staining was strongly reduced after preincubation of the antibody with an excess of the immunization peptide (Fig. 4*B*, *right panel*). The presence of EBBP in both the cytoplasm and the nucleus was also confirmed by Western blot analysis of cytoplasmic and nuclear fractions of HaCaT cells (not shown), although exact quantification was not possible due to the high insolubility of the protein. These results demonstrate that (i) the antibody is suitable for the specific detection of EBBP by Western blotting or immunofluorescence and that (ii) endogenous and recombinant EBBPs are mainly localized in the cytoplasm of COS-1 and HaCaT cells.

Serum, KGF, and EGF Maintain High Levels of EBBP Protein in Serum-starved HaCaT Cells—To determine a possible regulation of *ebbp* expression by KGF or EGF at the protein level, serum-starved HaCaT cells were treated with KGF, EGF, or serum using the conditions described for the RNA experiments (Fig. 1). No difference in the expression of EBBP protein was observed within 6–24 h after growth factor or serum addition (Fig. 5). This finding demonstrates that the initial upregulation and subsequent down-regulation of EBBP mRNA expression upon addition of KGF or EGF does not affect the levels of EBBP protein within this period. However, we observed significant differences between control and FCS/EGF/KGF-stimulated cells after longer incubation periods. Thus, a longer starvation of the cells caused a strong down-regulation of EBBP protein expression (compare control bands after 6, 24, 36, and 48 h in Fig. 5). Within this time frame the down-regulation was prevented by addition of serum and EGF and to a lesser extent also by KGF. Thus, the regulation of *ebbp* mRNA differed from that of the corresponding protein, demonstrating that *ebbp* expression is not only regulated at the transcriptional but also at the post-transcriptional level.

EBBP Expression in Human Skin—To determine the *in vivo* expression of EBBP, we stained frozen sections from human skin with our affinity purified EBBP antibody (Fig. 6*A*). EBBP was detected throughout the epidermis of human skin, but the expression in the basal layer was generally higher than in the keratin 10-positive suprabasal layers (Fig. 6, *B* and *C*). However, EBBP levels increased again in cells of the cornified layer. Weak signals were also observed in the dermal layer. The specificity of the staining was confirmed by preincubation of the antibody with the immunization peptide. In this case only a very weak background signal was observed, whereas preincubation with a non-related peptide had no effect (data not shown).

EBBP Expression during Cutaneous Wound Repair—To determine a possible role of EBBP in cutaneous wound repair, we prepared RNA and protein lysates from full-thickness excisional mouse wounds at different stages of the healing process, and analyzed the levels of EBBP mRNA and protein by RNase protection assay and Western blotting. As shown in Fig. 7*A*, the levels of EBBP mRNA decreased slightly within 3 days after injury, and slowly increased again at day 7 after wounding. However, the mRNA levels were still lower than in nonwounded skin at day 13 after injury when the wound was fully healed. In a Western blot analysis of skin and wound lysates, a major protein with an apparent molecular weight of 75,000 was detected with the EBBP antibody. The size of this protein was comparable with the size of the major protein found in lysates of COS-1 cells after transfection with a murine EBBP expression vector (data not shown). This band was not detected when
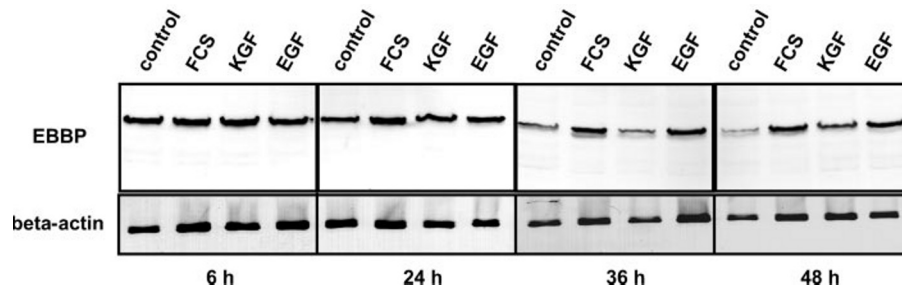
EBBP and Keratinocyte Differentiation

FIG. 5. **EBBP protein levels depend on the presence of serum, KGF, or EGF.** Confluent HaCaT cells were serum-starved for 24 h and stimulated with FCS, KGF, or EGF. Cellular lysates were prepared at the indicated time points and samples of 40 μg of total cellular protein were analyzed for EBBP expression by Western blotting using the polyclonal EBBP antibody. 20 μg of the same lysates were analyzed for β-actin expression (*lower panel*).
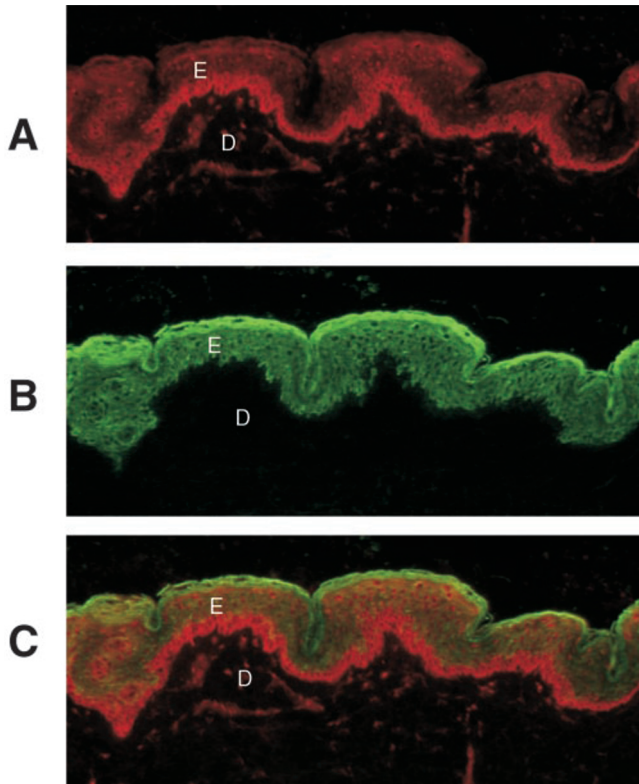

FIG. 6. **EBBP protein is predominantly expressed in undifferentiated keratinocytes of human skin.** Indirect immunofluorescence staining of acetone-fixed frozen sections from normal human skin using the EBBP antibody (*A*), the K10 antibody (*B*), or both (*C*) and Cy3^{TM}-coupled (*red*) or fluorescein isothiocyanate-coupled (*green*) secondary antibodies. Cells expressing both EBBP and K10 appear *yellow* (*C*). *D*, dermis; *E*, epidermis. Magnification: $\times$10.

the antibody was pretreated with the immunization peptide (data not shown). The bands migrating at an apparent size of about 60 kDa are unspecific, because they were also detected when the membrane was only incubated with the secondary antibody (not shown). Equal loading was controlled by Ponceau S staining of the membrane after blotting (not shown). As an additional control, the same lysates were analyzed for the presence of β-actin (Fig. 7*B*). Interestingly, the levels of EBBP protein declined rapidly after wounding, and the 75-kDa EBBP protein was undetectable within the first 2 days after injury. The down-regulation of EBBP protein expression after wounding occurred much faster compared with the down-regulation of the RNA, and the reduction of the protein was also more severe. These findings demonstrate once more that EBBP expression is also regulated at the post-transcriptional level.

Using immunohistochemistry, we found a strong expression of EBBP throughout the thin murine epidermis and in hair follicles of non-wounded skin (Fig. 7*C*). However, the signal intensity was significantly reduced in the hyperthickened wound epidermis, and only the most suprabasal, redifferentiating keratinocytes within the wound expressed significant amounts of EBBP (Fig. 7*D*). Similar as in human skin, EBBP expression was also observed in cells of the dermis.

Overexpression of EBBP Does Not Affect HaCaT Cell Proliferation—To gain insight into the function of EBBP in keratinocytes we generated stable HaCaT cell lines that overexpress human EBBP-HA or non-tagged EBBP. These cells were analyzed for EBBP expression under exponential growth conditions. Three cell lines were obtained that express EBBP-HA at similar or higher levels compared with the endogenous EBBP protein (Fig. 8*A*). The EBBP-HA expression of two other cell lines was much lower. A double band was observed for both the endogenous EBBP and for the EBBP-HA protein, most likely due to post-translational modification.

To determine whether the overexpression of EBBP-HA affects keratinocyte proliferation, an equal number of vector-transfected and EBBP-HA-transfected cells was seeded into Petri dishes and the cell numbers were determined at different time points. Surprisingly, the plating efficiency of the EBBP-HA-transfected cells was reduced by about 30% compared with vector-transfected cells. Therefore, the number of seeded cells had to be appropriately adjusted to obtain an identical number of attached cells in each dish. As shown in Fig. 8*B*, the proliferation rate of the EBBP-HA transfected cells was identical to that of vector-transfected cells, demonstrating that EBBP-HA overexpression does not affect HaCaT cell proliferation under exponential growth conditions.

EBBP Overexpression Induces Keratinocyte Differentiation—A possible explanation for the reduced plating efficiency of EBBP-transfected HaCaT cells is partial differentiation. Therefore, we determined whether EBBP-HA affects keratinocyte differentiation in exponentially growing or quiescent HaCaT cells. Consistent with the normal proliferation rate of subconfluent cells in the presence of serum, overexpression of EBBP did not affect expression of the differentiation-specific keratin 10 under these conditions (data not shown).

As a next step, we determined whether EBBP affects differentiation under differentiation permissive conditions. For this purpose, EBBP-HA- and vector-transfected cells were grown to confluence and serum-starved overnight. As shown in Fig. 9, *upper panel*, the levels of endogenous EBBP mRNA were reduced in the EBBP-HA overexpressing HaCaT cells under these conditions. A similar reduction was also seen at the protein level (Fig. 9, *second panel*). Most interestingly, significantly higher levels of K10 were detected in the EBBP-HA-transfected cells compared with control cells, and the levels of K10 correlated with the levels of EBBP-HA. These results were reproduced with the cells that overexpress the non-tagged version of EBBP, demonstrating that the effect is not due to the

FIG. 7. **EBBP expression during cutaneous wound repair in mice.** Mice were wounded as described under "Experimental Procedures" and sacrificed at different time points after injury. *A*, samples of 20 μg of total cellular RNA from normal and wounded skin were analyzed by RNase protection assay for the presence of EBBP and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) mRNAs. 20 μg of tRNA was used as a negative control. 1000 cpm of the hybridization probes were loaded in the lanes labeled "*probe*" and used as a size marker. *B*, samples of 60 μg of total protein from normal and wounded mouse skin were analyzed by Western blotting for the presence of EBBP. The 75-kDa EBBP protein is indicated with an *arrow*. The bands at about 60 kDa result from the secondary antibody (not shown). The same protein lysates (20 μg) were used for a Western blot with an anti-β-actin antibody. The experiment was reproduced with protein lysates from an independent wound healing experiment. *C*, acetic acid/ethanol-fixed paraffin sections (6 μm) from normal murine back skin (*C*) or from the middle of a 5-day-old full-thickness excisional mouse wound (*D*) were analyzed by indirect immunofluorescence using the EBBP antibody. *E*, epidermis; *D*, dermis; *G*, granulation tissue; *HE*, hyperproliferative epithelium; *HF*, hair follicle. Magnification: ×10.
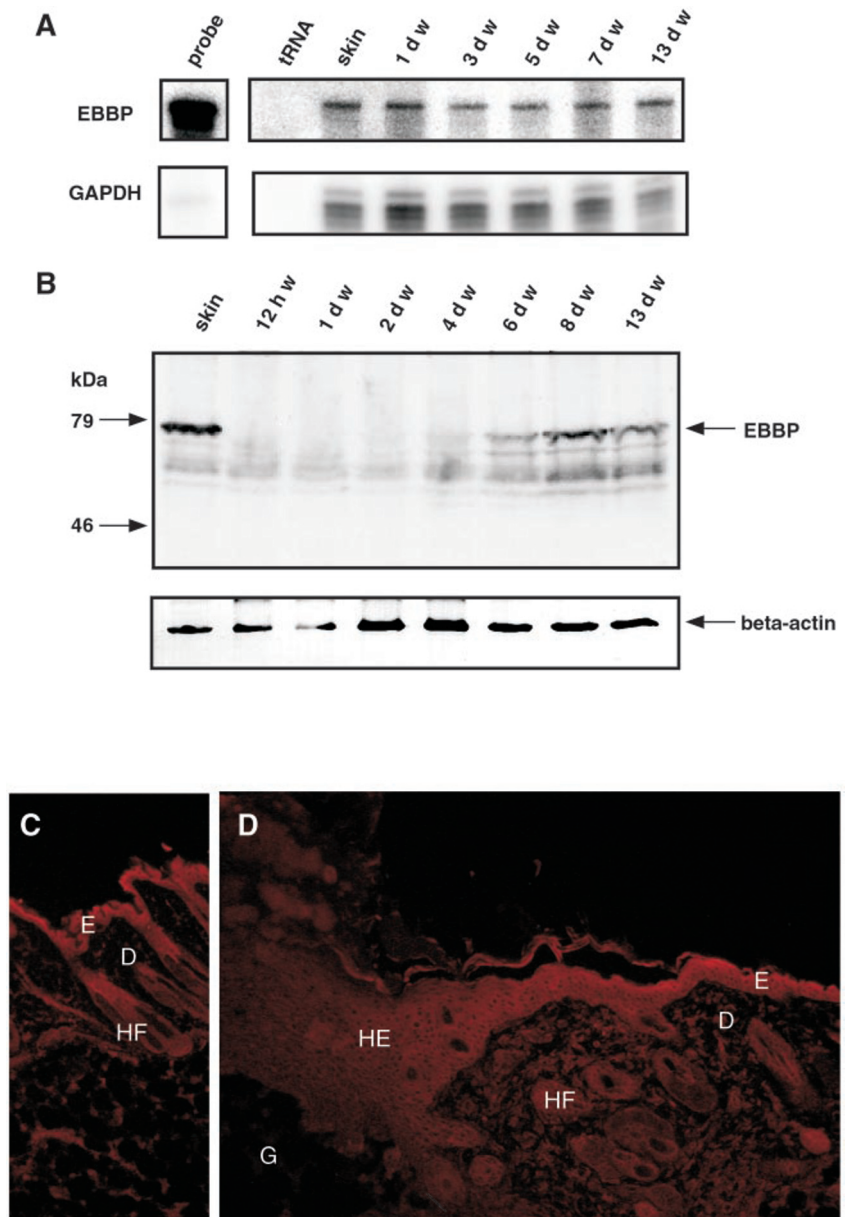
presence of the epitope tag (data not shown). Expression of involucrin, a protein that is expressed at later stages of keratinocyte differentiation (1), was also elevated in the EBBP-HA-transfected cells, but to a lesser extent compared with K10. Expression of keratin 6, a marker of abnormal differentiation (1), was also increased in the EBBP-HA-overexpressing cells. The retinoblastoma (RB) protein is the product of a tumor suppressor gene, which can bind and inhibit members of the E2F family of transcription factors in a phosphorylation-dependent manner and thereby prevent the progression through the cell cycle (28). Hyperphosphorylated, inactive Rb migrates slower on SDS-PAGE than the hypophosphorylated, active form. In lysates from EBBP overexpressing cells a down-regulation of both forms of Rb was detected in comparison to vector-transfected cells (Fig. 9). This down-regulation of both forms is characteristic for differentiated cells, and has also been observed during suspension-induced terminal differentiation of primary human epidermal keratinocytes (29). Taken together, these results demonstrate that overexpression of EBBP in HaCaT cells induces early differentiation in confluent, serum-starved cultures.

DISCUSSION

In this study we describe a novel KGF-regulated gene which encodes the EBBP protein. The *ebbp* gene has recently been described as a gene that is regulated by estrogen in mammary epithelial cells stably transfected with an estrogen receptor cDNA (16). It is ubiquitously expressed in various human tissues (16). However, nothing is as yet known about the function of the encoded protein.

EBBP contains several interesting domains (Figs. 2 and 3*A*). An acidic domain at the amino terminus is followed by two B boxes and three α-helices which may form a coiled-coil domain. Two motifs within these α-helices, F*XX*LL and L*XX*LL, have been identified as nuclear receptor interaction domains in transcriptional co-factors such as TIF1, NSD-1, and CBP (30, 31). At the carboxyl terminus, an RFP or B30.2 domain was detected. This domain composition is characteristic of a class of proteins called RING B-box coiled-coil (RBCC) proteins (26) or tripartite motif (TRIM) proteins (23). Although EBBP does not contain a RING finger, the other domains are present in this protein. RING fingers and B boxes are cysteine- and histidine-rich zinc-binding motifs mediating protein-protein interac-

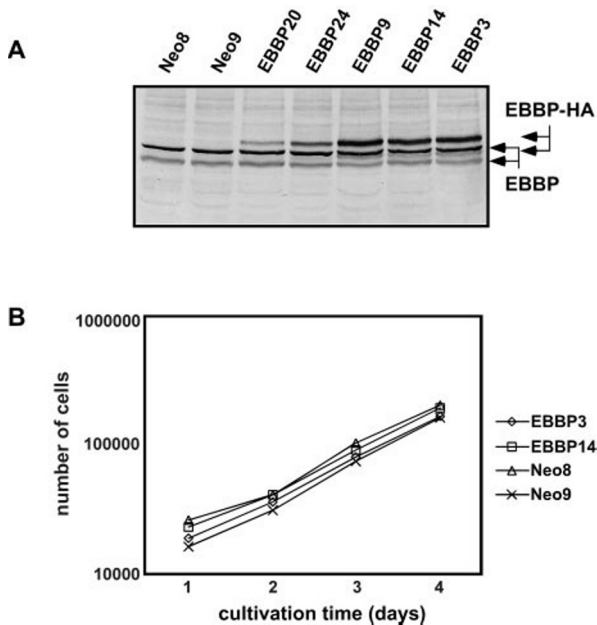*EBBP and Keratinocyte Differentiation*

FIG. 8. **Overexpression of EBBP in HaCaT cells does not affect proliferation.** HaCaT cells were stably transfected with an EBBP-HA expression vector and with a plasmid conferring G418 resistance (EBBP) or with the resistance plasmid and the empty expression vector (Neo). *A*, samples of 60 μg of total cellular protein of exponentially growing cells were analyzed by Western blotting for the expression of endogenous and recombinant EBBP proteins. The slowly migrating double band (*upper arrow*) corresponds to the recombinant, tagged protein (EBBP-HA), the other double band to endogenous EBBP (EBBP). *B*, EBBP-HA-transfected and control cells were seeded into 3.5-cm Petri dishes. At the indicated time points the cells were trypsinized and counted in triplicate. The growth medium was changed every 2 days. Results were reproduced in three independent experiments.

tions. The B box is important for homo- and heterodimerization of the RBCC proteins (23, 26).

The biological functions of RBCC proteins have been only partially characterized. Most of them are involved in the regulation of cell growth and differentiation during development and in the adult organism. Most interestingly, mutations in RBCC and other B box containing proteins have been found in several human diseases. For example, a translocation generating a fusion protein between the PML gene product and the retinoic acid receptor α is associated with promyelocytic leukemia, and the transcriptional co-activator TIF1 was found to be fused to the tyrosine kinase RET in childhood thyroid carcinomas. Mutations in the B30.2 domain of pyrin have been found in familial Mediterranean fever, and mutations in the B30.2 domain of midin cause Opitz syndrome, a multiple congenital anomaly manifested by abnormal closure of midline structures (32). These findings suggest that RING finger and B box proteins regulate crucial cellular functions.

In this study we have characterized the EBBP protein and we studied its regulation in keratinocytes. We demonstrate that the human EBBP protein present in COS-1 cells and HaCaT cells has an apparent molecular weight of 75,000, a finding which is consistent with previous data obtained with HepG2 cells (16). Since the calculated molecular weight is only 64,000, it seems likely that the protein is post-translationally modified. This hypothesis is supported by the presence of several putative phosphorylation sites in the protein, and by our observation that both endogenous EBBP and EBBP-HA often run as double bands in SDS gels.

In the skin, EBBP was strongly expressed in the proliferation-competent, non-differentiated keratinocytes of the basal layer. These cells are also targets of KGF and EGF action (8,
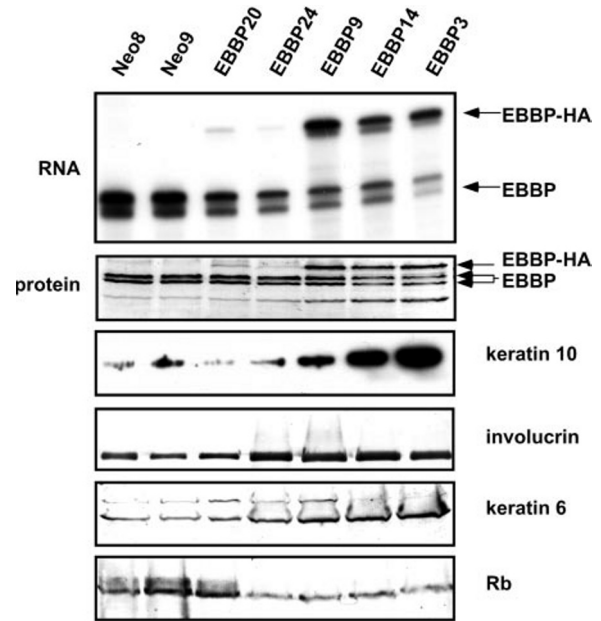

FIG. 9. **Expression of differentiation-specific proteins in EBBP-transfected HaCaT cells.** *Top panel*: RNase protection assay of 20 μg of total RNA using a ^{32}P-labeled antisense probe that can distinguish between the endogenous EBBP mRNA (*lower arrow*) and the mRNA encoding the tagged fusion protein (*upper arrow*). *Second through sixth panels*: immunoblots of total cellular proteins from confluent HaCaT cells stably overexpressing EBBP-HA and from control cells that had been serum-starved overnight. Samples of 60 μg of total protein were used for the EBBP Western blot, 10 μg for K10, 30 μg for involucrin, 30 μg for K6, and 50 μg for Rb.

33), indicating that the maintenance of high levels of EBBP by KGF and EGF observed *in vitro* might also be important in normal skin *in vivo*. However, despite the high levels of KGF and EGF receptor ligands in skin wounds (8, 9, 34, 35), expression of EBBP was strongly down-regulated in the hyperthickened wound epithelium, indicating the presence of other, as yet unidentified factors in a wound that suppress *ebbp* gene expression.

The hyperproliferative wound epithelium is characterized by the presence of rapidly proliferating keratinocytes with reduced differentiation capacity (2). The significantly reduced levels of EBBP seen in these cells suggest a role of EBBP in the regulation of keratinocyte proliferation and/or differentiation. This hypothesis is supported by preliminary results from our laboratory demonstrating that EBBP levels are also reduced in the hyperthickened epidermis of psoriatic patients, and particularly in the keratinocytes of basal cell carcinomas. Most importantly, the results obtained with our HaCaT cells that overexpress EBBP provide functional evidence for this hypothesis. Whereas proliferation was not altered in these cells, EBBP overexpression strongly stimulated the early differentiation process. This finding suggests that the presence of high levels of EBBP in keratinocytes is important for the onset of keratinocyte differentiation under permissive conditions. Thus, EBBP could play an important role in the induction of the differentiation pathway, a finding that is consistent with the common roles of RBCC members and related proteins in the regulation of cell growth and differentiation during development and in the adult organism. In addition to a possible role of EBBP in early differentiation, the strong EBBP staining of the cornified layer indicates an additional role of EBBP in late differentiation. In a hyperproliferative epithelium as seen in wounded and psoriatic skin and in basal cell carcinomas, differentiation is inhibited, and down-regulation of *ebbp* expression might be an important prerequisite for maintaining the cells in a non-differentiated stage.

The molecular mechanisms of EBBP action are as yet unknown. However, the presence of several protein domains that are characteristic for transcriptional regulators, suggests that EBBP can directly or indirectly modulate gene transcription. This hypothesis is supported by the recently observed DNA-binding capacity of EBBP in the presence of all cellular proteins.[2] The presence of two B boxes in conjunction with the coiled-coil domain in the EBBP protein suggests that it acts as a homo- or heterodimer or -multimer as shown for other TRIM family members (23). Indeed, preliminary co-precipitation experiments revealed that EBBP does indeed form homomultimers.[3] In addition, EBBP is likely to interact with other, as yet unidentified proteins, as suggested by the presence of several protein-protein interaction domains. In particular, the L*XX*LL domains are responsible for interaction with nuclear receptors such as the retinoic acid receptors (30, 31). These interacting proteins could also be responsible for the observed DNA-binding capacity, since EBBP lacks a DNA-binding domain. The predominant cytoplasmic localization of EBBP suggests that it retains possible DNA-binding interaction partners in this compartment, thereby inhibiting their function.

In summary, we have identified EBBP as the product of a novel KGF-regulated gene. Its expression in the KGF-responsive keratinocytes of normal skin as well as the induction of the differentiation program in EBBP-transfected HaCaT cells suggests that it mediates, at least in part, the differentiation stimulating activity of KGF under differentiation-permissive conditions (14, 15). Since EBBP is also expressed at high levels in various other epithelial cells,[2] it will be important to determine whether it also affects the balance between proliferation and differentiation of these cell types *in vitro*, as well in development, repair, and/or disease.

REFERENCES

1. Fuchs, E. (1990) *J. Cell Biol.* **111**, 2807–2814
2. Martin, P. (1997) *Science* **276**, 75–81
3. Carter, D. M. (1987) in *Dermatology in General Medicine* (Fitzpatrick, T. B., Eisen, A. Z., Wolff, K., Freedberg, I. M., and Austen, K. F., eds) 3rd Ed., pp. 759–765, McGraw-Hill, New York
4. Christophers, E., and Krueger, G. G. (1987) in *Dermatology in General Medicine* (Fitzpatrick, T. B., Eisen, A. Z., Wolff, K., Freedberg, I. M., and Austen, K. F., eds) 3rd Ed., pp. 461–491, McGraw-Hill, New York
5. Rubin, J. S., Osada, D. P., Chedid, M., Miki, T., Ron, D., Cheon, G., Taylor, W. G., Fortney, E., Sakata, H., Finch, P. W., and LaRochelle, W. J. (1995) *Cell Biol. Intern.* **19**, 399–411
6. Miki, T., Bottaro, D. P., Fleming, T. P., Smith, C. L., Burgess, W. H., Chan, A. M., and Aaronson, S. A. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89**, 246–250
7. Werner, S. (1998) *Cytokine Growth Factor Rev.* **9**, 153–165
8. Werner, S., Peters, K. G., Longaker, M. T., Fuller-Pace, F., Banda, M., and Williams, L. T. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89**, 6896–6900
9. Marchese, C., Chedid, M., Dirsch, O. R., Csaky, K. G., Santanelli, F., Latini, C., LaRochelle, W. J., Torrisi, M. R., and Aaronson, S. A. (1995) *J. Exp. Med.* **182**, 1369–1376
10. Werner, S., Smola, H., Liao, X., Longaker, M. T., Krieg, T., Hofschneider, P. H., and Williams, L. T. (1994) *Science* **266**, 819–822
11. Tsuboi, R., Sato, C., Kurita, Y., Ron, D., Rubin, J. S., and Ogawa, H. (1993) *J. Invest. Dermatol.* **101**, 49–53
12. Rubin, J. S., Osada, H., Finch, P. W., Taylor, W. G., Rudikoff, S., and Aaronson, S. A. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86**, 802–806
13. Hines, M. D., and Allen-Hoffmann, B. L. (1996) *J. Biol. Chem.* **271**, 6245–6251
14. Marchese, C., Rubin, J., Ron, D., Faggioni, A., Torrisi, M. R., Messina, A., Frati, L., and Aaronson, S. A. (1990) *J. Cell. Physiol.* **144**, 326–332
15. Marchese, C., Sorice, M., De Stefano, C., Frati, L., and Torrisi, M. R. (1997) *Cell Growth Differ.* **8**, 989–997
16. Liu, H.-L. C., Golder-Novoselsky, E., Seto, M. H., Webster, L., McClary, J., and Zajchowski, D. A. (1998) *Mol. Endocrinol.* **12**, 1733–1748
17. Boukamp, P., Petrussevska, R. T., Breitkreutz, D., Hornung, J., Markham, A., and Fusenig, N. E. (1988) *J. Cell Biol.* **106**, 761–771
18. Wakita, H., and Takigawa, M. (1999) *J. Biol. Chem.* **274**, 37285–37291
19. Andrews, N. C., and Faller, D. V. (1991) *Nucleic Acids Res.* **19**, 2499
20. Chomczynski, P., and Sacchi, N. (1987) *Anal. Biochem.* **162**, 156–159
21. Frank, S., and Werner, S. (1996) *J. Biol. Chem.* **271**, 24337–24340
22. Madlener, M., and Werner, S. (1997) *Gene* (*Amst.*) **202**, 75–81
23. Reymond, A., Meroni, G., Fantozzi, A., Merla, G., Cairo, S., Luzi, L., Riganelli, D., Zanaria, E., Messali, S., Cainarca, S., Guffanti, A., Minucci, S., Pelicci, P. G., and Ballabio, A. (2001) *EMBO J.* **20**, 2140–2151
24. Igarashi, M., Finch, P. W., and Aaronson, S. A. (1998) *J. Biol. Chem.* **273**, 13230–13235
25. Rheinwald, J. G., and Green, H. (1977) *Nature* **265**, 421–424
26. Borden, K. L. B. (1998) *Biochem. Cell Biol.* **76**, 351–358
27. Henry, J., Mather, I. H., McDermott, M. F., and Pontarotti, P. (1998) *Mol. Biol. Evol.* **15**, 1696–1705
28. Weinberg, R. A. (1995) *Cell* **81**, 323–330
29. Harvat, B. L., Wang, A., Seth, P., and Jetten, A. M. (1998) *J. Cell Sci.* **111**, 1185–1196
30. Heery, D. M., Kalkhoven, E., Hoare, S., and Parker, M. G. (1997) *Nature* **387**, 733–736
31. Huang, N., vom Baur, E., Garnier, J.-M., Lerouge, T., Vonesch, J.-L., Lutz, Y., Chambon, P., and Losson, R. (1998) *EMBO J.*, **17**, 3398–3412
32. Torok, M., and Etkin, L. D. (2001) *Differentiation* **67**, 63–71
33. King, L. E., Jr., Gates, R. E., Stoscheck, C. M., and Nanney, L. B. (1990) *J. Invest. Dermatol.* **95**, 10S–12S
34. Todd, R., Donoff, B. R., Chiang, T., Chou, M. Y., Elovic, A., Gallagher, G. T., and Wong, D. T. (1991) *Am. J. Pathol.* **138**, 1307–1313
35. Marikowsky, M., Breuing, K., Liu, P. Y., Eriksson, E., Higashiyama, S., Farber, P., Abraham, J., and Klagsbrun, M. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90**, 3889–3893

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[2] H.-D. Beer, unpublished data.

[3] C. Munding, unpublished data.