Transcription Factors C/EBPα, C/EBPβ, and CHOP (Gadd153) Expressed During the Differentiation Program of Keratinocytes *In Vitro* and *In Vivo*

Edward V. Maytin*† and Joel F. Habener†‡

*Department of Dermatology and †Laboratory of Molecular Endocrinology, Massachusetts General Hospital, ‡Howard Hughes Medical Institute, and Harvard Medical School, Boston, Massachusetts, U.S.A.

CCAAT-enhancer binding proteins (C/EBP) are basic region/leucine zipper (bZIP) transcription factors select-ively expressed during the differentiation of liver, adipose tissue, blood cells, and the endocrine pancreas. Here we show that C/EBP isoforms are differentially expressed in the skin. BALB/MK keratinocytes incubated in 0.12 mM calcium medium undergo a differentiation program fea-turing growth-arrest at 24–48 h, keratin K10 gene expres-sion beginning at 24 h, and apoptosis commencing at 48 h. Within this framework, western immunoblot analysis and immunohistochemistry reveal that C/EBPα increases 5-fold at 1–2 d and remains elevated, C/EBPβ rises 2-fold at 2–4 d and gradually falls, and CHOP rises 9-fold in the first 24 h then returns rapidly to baseline. Several products of alternative translation are observed in BALB/MK cells, i.e., 42 kDa and 30 kDa forms of C/EBPα, and 32 kDa and 20 kDa forms of C/EBPβ. By immunohis-tologic examination of human, rat, and mouse skin, all three transcription factors are highly expressed within epithelial compartments in a spatially restricted distribu-tion. C/EBPα is concentrated in the upper epidermis in a predominantly cytoplasmic location within cells, whereas the highest levels of C/EBPβ and CHOP are seen in the mid-epidermis, mainly within nuclei. High levels of C/EBPβ and CHOP (but not C/EBPα) are also observed in hair follicles and sebaceous glands. The identity of these factors in the epidermis is confirmed by western immunoblot analyses. In summary, C/EBP are expressed in a differentiation-associated manner in the skin, and may play an important role in regulating one or more aspects of the epidermal differentiation program. *Key words: cell differentiation/skin. J Invest Dermatol 110:238–246, 1998*

The CCAAT-enhancer binding proteins (C/EBP) are a group of mammalian transcription factors, originally characterized by their sequence-specific binding to CCAAT motifs in DNA. They are the subject of considerable interest because of their increasingly appar-ent importance in the regulation of cellular differentiation (reviewed in McKnight, 1992). C/EBP are members of the bZIP class of leucine zipper transcription factors that also includes AP-1, CREB/ATF, PAR, and plant G-box proteins (reviewed in Lamb and McKnight, 1991; Vinson *et al*, 1993; Haas *et al*, 1995). The prototype member of the C/EBP family, first called CBP (Graves *et al*, 1986), then C/EBP (Landschulz *et al*, 1988), and finally C/EBPα, was isolated from rat liver nuclei where it was shown to regulate transcription of the albumin gene (Friedman *et al*, 1989) and other genes expressed in differentiated hepatocytes (Costa *et al*, 1988). Subsequently, other C/EBPα-related proteins named C/EBPβ, C/EBPγ, C/EBPδ, and C/EBPε were cloned (reviewed in McKnight, 1992). The newest member of the C/EBP family is called CHOP (<u>C</u>/EBP-<u>Ho</u>mologous <u>P</u>rotein) (Ron and Habener, 1992), and acts in most, but not all circumstances (Ubeda *et al*, 1996) as a dominant-negative inhibitor of DNA-binding when it is heterodimerized to another C/EBP partner. CHOP is identical to the growth-arrest and DNA damage-inducible protein gadd153 (Luethy *et al*, 1990).

Expression of the C/EBP isoforms appears to occur sequentially during the differentiation of cells. The best evidence for this idea comes from adipoblast cell lines differentiating in culture (e.g., 3T3-L1, F442 A, 30 A5). In this system, a unique time course of expression has been documented for each of the proteins C/EBPα, C/EBPβ, C/EBPδ, and CHOP (Cao *et al*, 1991; Ron and Habener, 1992). For example, C/EBPα is not expressed early in the differentiation of adipoblasts, but increases during the latter half of the 1 wk differentiation protocol (Cao *et al*, 1991) and decreases again if fully differentiated adipocytes are reverted to an undifferentiated state by exposure to TNFα (Ron *et al*, 1992). Other types of experiments also support a critical role for C/EBP in the differentiation program for fat cells. Ectopic overexpression of C/EBPα is capable of driving adipoblasts to the fully differentiated phenotype (Freytag *et al*, 1994; Lin and Lane, 1994), whereas C/EBPα anti-sense RNA expressed in the adipoblasts can block the normal program, both by preventing growth-arrest and by reducing expression of several adipose differentiation related genes (Lin and Lane, 1992). In contrast to C/EBPα, the species C/EBPβ, C/EBPγ, and C/EBPδ are expressed early in differentiation and then defervesce as C/EBPα begins to rise (Cao *et al*, 1991). Thus, evidence is accumulating that the different C/EBP genes are involved differentially in the temporal progression of progenitor cells along a differentiation pathway, presumably by individually modulating the transcription of genes crucial to achieving and/or maintaining the differentiated state.

This study is an examination of C/EBP and their potential role in the differentiation of the mammalian epidermis. The epidermis is an attractive system in which to study differentiation, because the constituent keratinocytes follow a temporally and spatially well-defined program of stratification. As cells migrate upward from the proliferative basal layer, they first undergo growth-arrest, then sequentially express a series of unique gene products (e.g., keratin K1, keratin K10, loricrin, filaggrin), and finally die by apoptosis to form the dead barrier layer (reviewed in Fuchs, 1990). The notion that C/EBP might play a role in epidermal differentiation is suggested by a report that briefly mentions the detection of C/EBPα in mammalian epidermis (Birkenmeier et al, 1989). A Drosophila homolog of C/EBP in the epidermis of the fly has also been described (Rorth and Montell, 1992); however, a detailed examination of the expression of C/EBP in the skin has been lacking up to now.

Here we explore a possible role for C/EBP in the process of epidermal differentiation. As an in vitro experimental model to approximate the keratinoctye differentiation program, we employed the BALB/MK cultured murine keratinocyte line (Weissman and Aaronson, 1983; Maytin et al, 1993). We find that when the calcium concentration is raised from 0.07 mM to 0.12 mM, BALB/MK cells undergo many aspects of the normal differentiation program in a manner similar to that reported for primary keratinocytes (Yuspa et al, 1989). Elevation of the calcium concentration [which may simulate an upward calcium gradient that exists in the epidermis in vivo (Yuspa et al, 1988)] induces growth-arrest followed by a temporal sequence of expression of different keratin isoforms (Marcelo et al, 1978; Hennings et al, 1980). The program eventually ends in apoptosis. We show that during differentiation of BALB/MK cells, three transcription factors (C/EBPα, C/EBPβ, and CHOP) undergo time-dependent increases in expression with unique temporal profiles. Furthermore, immunofluorescence studies on skin biopsy material from three different species demonstrate the presence of the C/EBP in keratinocytes in vivo, with each C/EBP family member displaying a distinctive gradient of expression within the epidermis. Together, these results suggest possible roles for the C/EBP family of transcription factors in regulating differentiation-specific genes in mammalian skin.

MATERIALS AND METHODS

Cell culture and differentiation protocol   BALB/MK cells (Weissman and Aaronson, 1983) were grown in low calcium medium (0.07 mM, special Eagle's MEM, Biofluids, Rockville, MD) to which 5 ng epidermal growth factor (Collaborative Research, Bedford, MA) per ml and 10% Chelex-treated serum (Marcelo et al, 1978) were added. For calcium step-up experiments, EMEM medium supplemented with calcium to 0.12 mM (Yuspa and Dlugosz, 1991) was added to cells that had been confluent for at least 2 d. Medium was changed daily for the duration of the experiment to minimize effects due to nutrient and growth factor depletion. Six-day time-course experiments were designed in reverse order (i.e., the switch to high-calcium occurred on the first day in the 6 d dish, on the second day in the 5 d dish, etc.), so that all dishes were maintained for an equal length of time and were processed together on the last day.

Transfection of cos cells   To provide well-defined proteins for verification of putative bands identified in BALB/MK lysates, cos-1 cells were transfected with pCDNA1 plasmids (In Vitrogen; San Diego, CA) containing cloned cDNA for each transcription factor. The vectors encoding full-length rat C/EBPα, full-length human C/EBPβ (LAP), and mouse CHOP have been described (Ron and Habener, 1992). To produce the 20 kDa form of C/EBPβ (LIP), the insert from the vector plasmid LIP-pRESET-A (gift of David Ron, NYU Medical School, New York) was excised by BamH1/Xho digestion and ligated into pCDNA1. Monolayers of cos-1 cells were transfected using the DEAE-Dextran method as described (Ausbel et al, 1992).

Immunocytochemistry   Primary rabbit anti-sera directed against C/EBPα, C/EBPβ, and CHOP/gadd153 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-keratin K10 was a mouse monoclonal antibody from Sigma (clone 8.6, cat. no. C-7284). For immunostaining experiments, BALB/MK cells were grown in 4 well or 8 well plastic chamber slides (Nunc, Naperville, IL) to allow simultaneous processing of cells at different stages of differentiation. Cells were fixed in 4% paraformaldehyde, then permeabilized in methanol followed by 0.1% Triton X-100 to achieve nuclear permeabilization (Collier and Schlesinger, 1986). Tissue specimens comprised

dorsal skin from rats and mice, and forearm skin from humans (excess surgical biopsy material). Specimens were frozen in OCT compound (Miles, Elkhart IN), cut into 5 μm sections, and permeabilized as above but without fixation prior to staining.

For slides stained by the peroxidase method, slides were blocked in 3% normal goat or donkey serum plus 40 μg avidin per ml, to reduce background. Specimens were incubated overnight with primary anti-sera (1:50) at 4°C, and blocked futher with 0.3% hydrogen peroxide and 0.002% biotin. After several phosphate-buffered saline washes, slides were incubated in the secondary antibody, a peroxidase-conjugated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA, 1:300 dilution). Subsequent incubations with Vector ABC complex and amino-ethylcarbazole were performed as described (Maytin et al, 1994). Slides were mounted in Glycergel (DAKO, Glostrup, Denmark).

For immunofluorescence studies, slides were blocked in 3% normal donkey serum, incubated overnight with primary anti-sera (1:50) at 4°C, rinsed in phosphate-buffered saline, and incubated for 4 h at room temperature in the secondary antibody, a Cy-3-conjugated donkey anti-rabbit or anti-mouse IgG (1:1500) from Jackson ImmunoResearch (West Grove, PA). After mounting in fluorescence mounting medium (Kirkegaard & Perry, Gaithersburg, MD), slides were viewed under a Nikon Optiphot-2 microscope equipped for epifluorescence viewing.

For staining of cell nuclei, tissue sections were incubated for 5 min in 20 μg per ml of the DNA-specific stain, bisbenzimide H 33258 (CalBiochem, San Diego, CA) prior to viewing at 450 nm on the epifluorescence microscope.

Proliferation and apoptosis assays   The following modifications to the 5′-bromodoxyuridine (BrdU) incorporation assay (RPN-20 kit, Amersham, Arlington Heights, IL) were crucial for reproducibility (Simonson et al, 1995). After fixation of the BrdU-labeled cells, a permeabilization step with 2 N HCl at 37°C for 1 h was performed. The slides were then soaked three times × 5 min in 0.1 M sodium tetraborate. Subsequent staining was carried out with reagents from NovoCastra/Vector (Burlingame, CA) using their NCL-BrdU monoclonal antibody and standard immunoperoxidase methods.

The terminal transferase dUTP nick end-labeling (TUNEL) assay was performed as described (Maytin et al, 1994). Alternatively, the ApopTag kit from Oncor (Gaithersburg, MD) gave similar results.

Western immunoblot analyses   For western blotting, rabbit polyclonal anti-sera were used in all cases. Two sets of anti-sera recognizing C/EBP isoforms were employed. In early experiments, anti-sera produced in our laboratory and directed against amino-terminal peptide epitopes were used. These anti-sera gave several nonspecific bands, in addition to the bands of interest on the blots. Subsequently, affinity-purified anti-sera against carboxy-terminal epitopes became available from Santa Cruz Biotechnology. These were used for later experiments because they gave qualitatively cleaner signals. Quantitatively, however, the results for C/EBP-containing bands were identical for the two types of anti-sera. [Pooled data (see **Fig 5**) were derived using both types of anti-sera.] The anti-K10 and anti-filaggrin anti-sera were gifts from Dr. Stuart Yuspa (NCI, Bethesda, MD), and the K14 anti-serum was obtained from Berkeley Antibody (Richmond, CA).

Cells from 100 mm dishes were trypsin-released, pelleted, lyzed in 200 μl of 9.5 M urea lysis buffer (Young et al, 1983), and ruptured with three 10 s bursts of an ultrasonic probe on ice. Aliquots of lysate were run on 12% sodium dodecyl sulfate-polyacrylamide gels alongside prestained molecular weight markers (Gibco BRL, Grand Island, NY), electrotransferred to nylon membranes (Immobilon, Millipore, Bedford, MA), reacted with anti-sera specific for the protein of interest (1:5000 dilution), and visualized using a chromophore-conjugated anti-IgG antibody and enhanced chemiluminescence reagents (ECL kit, Amersham) followed by exposure to X-Omat AR film (Kodak, Rochester, NY).

Uniformity of protein loading was assessed twice, initially prior to gel electrophoresis by testing 1 μl aliquots with an Amido Black assay (Sportsman and Elder, 1984), then again after completion of the western blot by staining the Immobilon membrane with 0.01% Amido Black/45% methanol/10% acetic acid. A digitizing camera (IS-1000 Digital Imaging System, Alpha Innotech, San Leandro, CA) was used to measure overall protein densities on Amido Black-stained membranes. For quantitation of western blots, the films were scanned on a two-dimensional laser scanning densitometer (Computing Densitometer from Molecular Dynamics, Sunnyvale, CA) and the integrated density after background subtraction was determined for each band of interest, as described (Maytin, 1992).

Computer-assisted quantitation of immunostained cells   Fluorescent or brightfield images on the Nikon Optiphot-2 microscope were captured and digitized using an Optronics Model TEC-470 CCD camera, connected to a MacIntosh 7100 PowerPC computer with a RasterOps framegrabber and IPLab Spectrum software (Signal Analytics, Vienna, Virginia). For fluorescent images
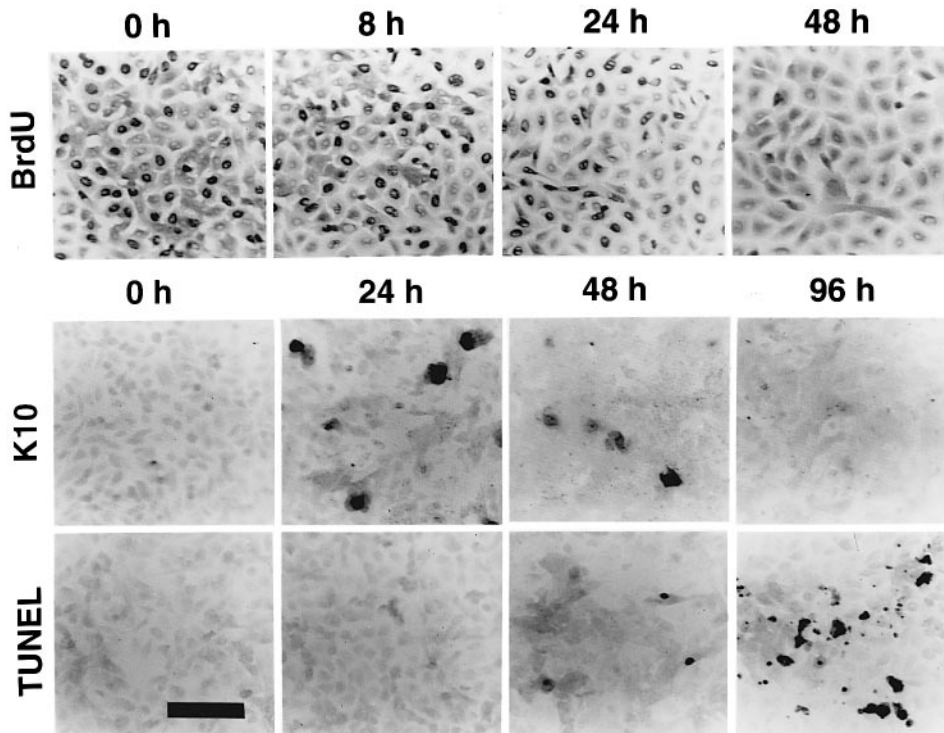
Figure 1. Assays for markers of proliferation (BrdU), differentiation-specific protein expression (keratin K10), and apoptosis (TUNEL) show the relative timing of growth-arrest, K10 expression, and apoptosis in BALB/MK keratinocytes. The cells were allowed to differentiate in 0.12 mM calcium for the times indicated above the parts. BrdU labeling identifies densely labeled nuclei in S-phase, immunoperoxidase staining of keratin K10 reveals stratifying islands that contain K10-positive cells, and TUNEL assay specifically labels cells undergoing apoptosis (with their dense, often fragmented nuclei). Photomicrographs shown here are representative; quantitative data can be found in **Table I**. *Scale bar*, 100 μm.

upon which quantitative intensity measurements were performed, care was taken to avoid saturation by adjusting contrast/brightness settings to bring all pixel values within the linear range of the CCD camera. For time-course measurements, the same contrast/brightness setting was used for all images in the series.

For the fluorescence intensity measurements shown in **Fig 7**(*a*), the tracing tool available within the IP Lab Spectrum program was used to divide the epidermis into eight equally sized polygonal areas. The polygons were drawn to encompass the eight or so cell layers present in each fluorescent specimen, as follows. First, the top and bottom boundaries of the living epidermis (dermal/epidermal and granular/corneal junctions) were determined by comparison to matched, phase-contrast images. Next, vertical lines were drawn at regular intervals along the specimen, to connect top and bottom boundaries. Each vertical line was divided into eight equal segments, and the endpoints of these segments were used as guides to draw eight horizontal polygons stacked one above the other (these polygons are referred to as "epidermal cell layers" in **Fig 7**). Within each polygon, the fluorescence intensity values of all pixels were averaged, and a background value (nonspecific signal in the dermis) was subtracted. Finally, the mean fluorescence intensity of each epidermal cell layer was converted to a percentage of the maximum value within that specimen.

Preparation of proteins from different layers of mouse epidermis Populations of epidermal cells, enriched in either upper or lower epidermal components, were prepared by modification of a technique normally used to prepare keratinocytes for primary culture (Marcelo *et al*, 1978; Maytin, 1992). Briefly, epidermal sheets from 20 newborn mice (FVB strain, Charles River, Wilmington, MA) were separated from the dermis after overnight digestion at 4°C in 0.25% trypsin, stirred vigorously in 10 ml EMEM medium to dislodge cells from the lower epidermis, and the cells layered over a Ficoll 400 step gradient (12, 15, 18, 20%) to remove contaminating fibroblasts. After centrifugation at 768 × *g* for 30 min at 4°C, cells from the 18% fraction were pelleted, resuspended in 400 μl of 9.5 M urea buffer, sonicated briefly, and frozen at –70°C. The latter was called the "lower fraction." Proteins from the "upper fraction" were prepared at 4°C by shredding the epidermal remnants with forceps, pressing out excess liquid with a pestle, adding 4 ml of 9.5 M urea buffer, and sonicating small aliquots in Eppendorf tubes until reasonably clear.

RESULTS

C/EBPα, C/EBPβ, and CHOP are preferentially expressed within stratifying islands in a keratinocyte model of differentiation To begin to characterize the C/EBP family of transcription factors in epidermal cells, we employed cultured BALB/MK keratinocytes that can be switched from a proliferating to a differentiating state by raising the calcium concentration of the medium. At 24 h after a switch from 0.07 mM to 0.12 mM calcium, BALB/MK cells began to stratify, forming scattered islands of cells that rose above the plane of the culture dish. To more carefully define the program of differentiation in BALB/MK cells, we performed assays to measure three facets of keratinocyte differentiation (**Fig 1**). Growth-arrest, as measured by BrdU incorporation into cell nuclei, occurred at 24–48 h after the calcium switch (**Fig 1**, *first row*). Upregulation of a prototypic differentiation-specific gene, keratin K10, occurred at 24 h; the K10 protein was found only within the stratifying islands (**Fig 1**, *second row*). As reported previously, the observed increase of K10 expression in mouse keratinocyte cultures was transient, gradually returning to baseline after 48 h (Yuspa *et al*, 1989). Apoptosis, the final component of differentiation, was measured using the TUNEL assay (**Fig 1**, *last row*). No significant apoptosis above background levels was observed until 48 h, after which time the number of apoptotic cells increased dramatically.

Within this framework of keratinocyte differentiation, we next examined the time courses for protein expression of the three C/EBP-family members (**Fig 2**). Representative low-power immunofluorescence views of BALB/MK cultures, stained at different times during the differentiation protocol (**Fig 2**), revealed bright objects representing stratified islands of cells that stained with the specific anti-sera. Specificity of staining was confirmed by the absence of bright signals in parallel cultures developed in preimmune sera (not shown). Compared with control cultures at 0 h, the levels of C/EBPα expression rose significantly within 24 h of the calcium switch, and remained
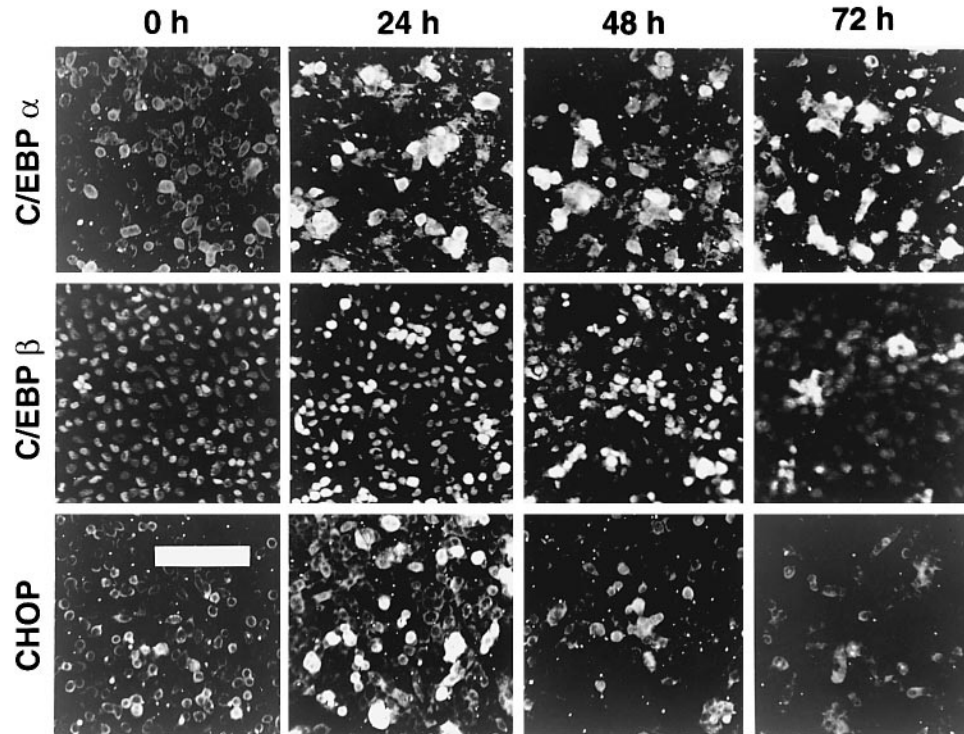
Figure 2. Time courses for expression of C/EBPα, C/EBPβ, and CHOP show that high levels of the transcription factors are found only in the differentiating islands that develop in BALB/MK cultures exposed to high calcium for 24 h or more. BALB/MK keratinocytes were allowed to differentiate for 24–48 h in 0.12 mM calcium medium, then were fixed and immunostained with primary anti-sera to C/EBPα, C/EBPβ, or CHOP, followed by a secondary antibody conjugated to the fluorescent dye, Cy3. A representative photomicrograph is shown for each time point indicated along the top. More quantitative data can be found in **Table I**. *Scale bar*, 100 μm.

elevated thereafter (**Fig 2**, *first row*). C/EBPβ rose at first, then began to fall by 72 h (**Fig 2**, *second row*). CHOP displayed the most transient change, rising and falling abruptly over the first 48 h (**Fig 2**, *third row*).

Close-up examination of cultures after fixation and staining with specific anti-sera (**Fig 3**), reconfirmed that the level of each transcription factor was specifically elevated within the islands. Comparison of the immunofluorescence patterns (**Fig 3***A*, *C*, *E*) with the corresponding phase-contrast images (**Fig 3***B*, *D*, *F*), demonstrated this selective increase in expression of C/EBPα, C/EBPβ, and CHOP within the stratifying islands relative to the surrounding monolayer. Notably, the intracellular distribution for each protein differed. C/EBPα was predominantly found in a cytoplasmic location. C/EBPβ was almost entirely nuclear. CHOP showed an intermediate pattern, with cytoplasmic signals in nearly every cell, along with nuclear staining in many of the cells.

The changes illustrated in **Figs 1–3** were substantiated in **Table I**, a presentation of quantitative results from these experiments. From these data, the initial increases in C/EBPα, C/EBPβ, and CHOP appeared to slightly precede or coincide with growth-arrest and with the onset of K10 expression. Apoptosis, on the other hand, began to increase at a time when levels of both CHOP and C/EBPβ were falling, relative to the persistent elevation of C/EBPα.

Western immunoblot analyses of proteins in BALB/MK cells show unique temporal profiles for C/EBPα, C/EBPβ, and CHOP during differentiation   To attain more quantitative information about the proteins detected by immunohistochemistry, we performed western analyses of proteins obtained from BALB/MK cells differentiating for various lengths of time (**Fig 4**). For each transcription factor, several proteins were anticipated because in addition to the full-length products, shorter translation products derived from alternative ribosomal translation sites within the mRNA have been described for C/EBPα and C/EBPβ (reviewed in An *et al*, 1996). To sort these out, well-defined protein standards and several anti-sera were used. As protein standards, aliquots of *cos* cells transfected with plasmids encoding the transcription factor of interest were analyzed by electrophoresis
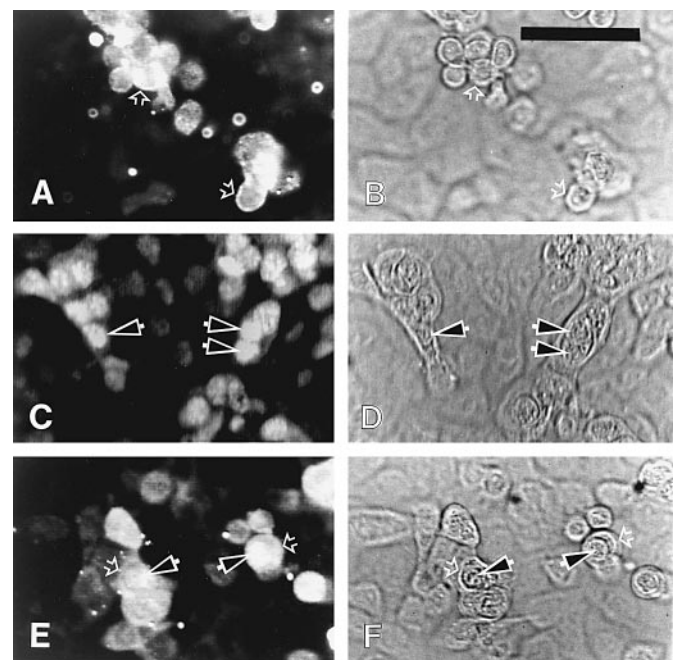

Figure 3. High-power immunofluorescence photomicrographs show that the intracellular amounts of C/EBPα (*A*, *B*), C/EBPβ (*C*, *D*), or CHOP (*E*, *F*), are preferentially elevated within cells of the stratifying islands. Fluorescence staining was performed as described in the legend to **Fig 2**. For each transcription factor, a pair of images (fluorescent on the left, phase contrast on the right) is presented from the same field in order to allow assessment of cytoplasmic localization (*open arrowheads*) *versus* nuclear localization (*closed arrowheads*) of the fluorescence signals. *Scale bar*, 50 μm.
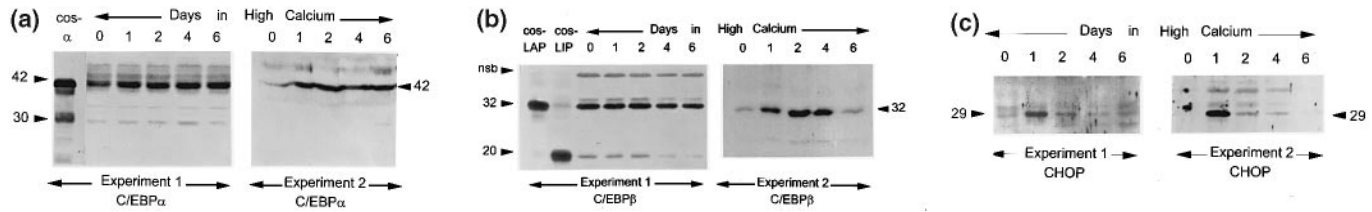
Figure 4. Western blots of whole–cell proteins from differentiating BALB/MK keratinocytes to demonstrate time-dependent changes in expression of C/EBPα, C/EBPβ, and CHOP. For each transcription factor, two experiments are shown, one using an anti-serum that recognizes the carboxy-terminus (experiment 1), and another that recognizes the amino-terminus (experiment 2) of each protein. In (a) and (b), several lanes contain standard proteins produced by transfecting cos cells with plasmid vectors expressing C/EBP isoforms. Relative molecular weights for each band of interest are indicated by *arrowheads*. (a) Stained with anti-C/EBPα anti-serum; the *cos* cells (cos–α) were transfected with full-length C/EBPα. (b) Stained with anti-C/EBPβ anti-serum; the *cos* cell lysates contained either full-length C/EBPβ (cos–LAP) or a shorter translational isoform of C/EBPβ (cos–LIP). nsb, nonspecific band. Note that in experiment 1 for C/EBPβ, a long exposure was used to demonstrate the minor 20 kDa band; therefore, changes in the major 32 kDa band cannot be well appreciated. (c) Stained with anti-CHOP anti-serum. A single product at 29 kDa is detected.

Table I. Quantitative assays for expression of C/EBP transcription factors, and for events in the keratinocyte differentiation program (proliferation, K10 gene expression, and apoptosis) reveal differences in their time courses of expression

	Time (h) of incubation in elevated calcium				
Assay (units)[a]	0	24	48	72	96
C/EBPα (positive islands per field)[b]	0.4 ± 0.7	11.1 ± 3.1	13.3 ± 2.0	11.8 ± 4.8	nd[f]
C/EBPβ (positive islands per field)[b]	0.7 ± 0.7	11.5 ± 3.3	13.4 ± 3.5	6.3 ± 1.2	nd
CHOP (positive islands per field)[b]	0.9 ± 1.0	9.0 ± 1.4	4.8 ± 2.3	0.2 ± 0.4	nd
BrdU (% of cells labeled)[c]	54.0 ± 6.9	32.1 ± 2.6	3.3 ± 2.2	nd[f]	nd
K10 (positive islands per field)[d]	0.8 ± 0.9	11.8 ± 4.2	4.1 ± 2.1	nd	0.8 ± 2.3
TUNEL (positive cells per field)[e]	4.0 ± 2.8	2.5 ± 1.5	34.7 ± 15.4	nd	124.0 ± 43

[a]Quantitative results from immunocytochemical staining are presented from experiments performed under similar conditions. Cells in 4 well, multichamber slides were kept at an elevated calcium concentration (0.12 mM) for the times indicated prior to fixation, immunostaining, and video capture in IPLab Spectrum. For each of the four conditions on a given slide, positively stained cells were counted from a sufficient number of low-power fields per condition to provide a reasonable measure of variance. The magnification (m) and number of fields counted (n) are listed below for each condition. Results are given as the mean ± SD.

[b]Cells expressing high levels of C/EBPα, C/EBPβ, and CHOP were located in clusters within stratifying islands. Individual cells were difficult to distinguish from one another, so positively stained islands were counted instead. A positive island is defined as three or more cells, to eliminate cell doublets (mitotic figures that appear to stain in some cases in a manner probably not related to differentiation); m = ×200; n = 10.

[c]BrdU was used to label proliferating cells in S-phase of the cell cycle; m = ×200; n = 8.

[d]Keratin K10-expressing islands; m = ×100; n = 50.

[e]TUNEL was used to identify apoptotic cells. The 96 h time point was from a separate experiment. m = ×400; n = 100.

[f]nd, not done.

alongside the BALB/MK lanes. Blots on the left side of **Fig 4** (experiment 1) were developed with anti-sera directed against the carboxy-terminal end of each protein, whereas anti-sera that recognize amino-terminal epitopes were used on the right (experiment 2). In the case of C/EBPα, both the 42 kDa full-length form and the 30 kDa form, a minor product that lacks the amino-terminal end, were detectable using the carboxy-terminal anti-serum (**Fig 4a**, *left*). The amino-terminal anti-serum detected only the 42 kDa form (**Fig 4a**, *right*), as expected. For both forms of C/EBPα, expression levels increased within 24 h and remained high for 6 d. C/EBPβ was also detectable as two alternative translation products (**Fig 4b**), the full-length 32 kDa form (LAP) and the shorter 20 kDa form (LIP). The 32 kDa form appeared as a doublet, in agreement with reports of post-translational modification, e.g., phosphorylation of C/EBPβ, in other other cell types (Metz and Ziff, 1991; Trautwein *et al*, 1993). Expression of both the 32 kDa and 20 kDa forms of C/EBPβ expression increased roughly in parallel, rising and falling over a 6 d period. CHOP was detected as a single translational product at 29 kDa, using either antibody (**Fig 4c**, *left* and *right*), in agreement with expected results (Ron and Habener, 1992). CHOP has sometimes been reported as a doublet on western immunoblots from several cell types, perhaps as a result of stress-related phosphorylation (Wang and Ron, 1996); we observed this in a few BALB/MK experiments as well (data not shown). CHOP, although quantitatively lower in abundance than C/EBPα or C/EBPβ, is particularly interesting because its induction is quite pronounced and defervesces rapidly. In **Fig 5(a)**, semiquantitative analysis of the data collected from many experiments showed that the increase in CHOP (10-fold) was the largest and most transient induction observed among the three transcription factors.

C/EBPα, C/EBPβ, and CHOP are detectable within mammalian epidermis, in locations that suggest a role for these factors in regulating normal differentiation   Although *in vitro* data from BALB/MK cells support a link between expression of certain C/EBP isoforms and the progression of normal keratinocyte differentiation, culture models can never perfectly mimic the actual situation *in vivo*. Therefore, evidence for *in vivo* expression of the C/EBP in real skin would be valuable. To obtain such evidence, we examined frozen sections of mouse, rat, and human skin by immunofluorescent staining (**Fig 6**). In all cases, the level at which transcription factor expression occurred could be determined by observing the sections under phase-contrast illumination (**Fig 6A**). The location of the basal layer, with its proliferative compartment, along with the nuclei of the suprabasal layers above it, could be confirmed by staining with H 33258, a DNA-specific stain (**Fig 6B**). Expression of the differentiation-specific gene, keratin K10, was seen in the suprabasal layers (**Fig 6C**), as described (Roop *et al*, 1988). Apoptosis was detectable only in cells of the granular layer, just below the stratum corneum (**Fig 6D**). Within this framework, relative expression of C/EBPα, C/EBPβ, or CHOP was evaluated. Elevated levels of C/EBPα were found in a predominantly cytoplasmic location within cells of the mid-stratum spinosum, later accumulating to high levels throughout cells of the upper spinosum, stratum granulosum, and statum corneum. This pattern was consistently seen in human (**Fig 6E**), rat (**Fig 6F**), and mouse (**Fig 6G**) epidermis. C/EBPβ had a different pattern, beginning as a pronounced cytoplasmic staining in the basal layer, changing to a predominantly nuclear location in the middle to upper stratum spinosum, and disappearing thereafter (**Fig 6H**). CHOP expression resembled that of C/EBPβ; CHOP staining in the lower epidermis was cytoplasmic and perinuclear, with
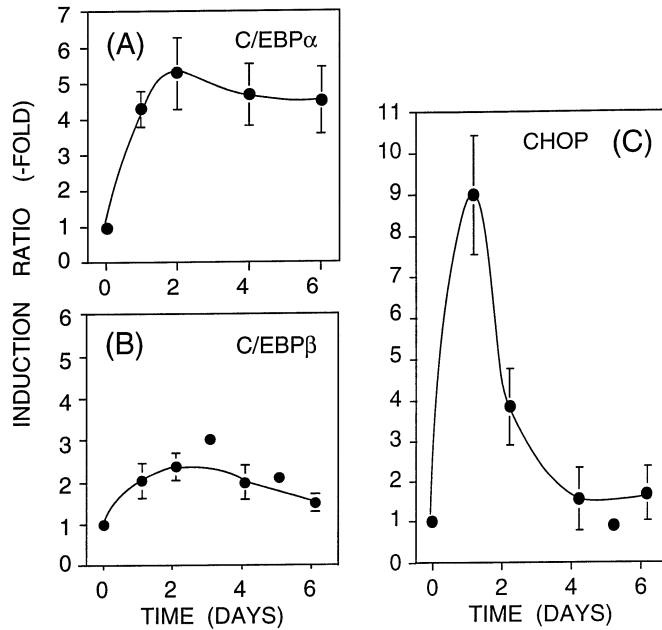
Figure 5. Quantitative time courses for the expression of the three full-length transcription factors in differentiating BALB/MK cells. (*A*) C/EBPα, (*B*) C/EBPβ, and (*C*) CHOP. Cells were maintained in 0.12 mM calcium for the times indicated prior to lysis and ECL-western analysis. Relative increases in expression of full-length C/EBPα, C/EBPβ, and CHOP were determined by scanning densitometry. Results are given as the relative induction of differentiated *versus* control cells. Multiple western blots were pooled for this analysis; each point represents the mean ± SEM of 5–8 experiments.

a gradual increase in nuclear staining at higher levels (**Fig 6K**). Thus, CHOP and C/EBPβ were expressed in all living layers of the epidermis, whereas C/EBPα was expressed predominantly in the granular layer and persisted into the stratum corneum.

C/EBPβ and CHOP (but not C/EBPα) were also detected in epidermal appendages. Relatively high levels of C/EBPβ were found in the outermost layer of the sebaceous glands (**Fig 6I**), and in both the root sheaths (**Fig 6I**) and the bulb matrix regions of hair follicles (**Fig 6J**). Likewise, high levels of CHOP were seen in sebaceous glands and in hair follicles (**Fig 6L**). Normal sera controls (**Fig 6M**) gave essentially no fluorescent signal, confirming the specificity of the immunostaining.

To further compare expression patterns in the skin, fluorescent intensity profiles for C/EBPα, C/EBPβ, and CHOP were calculated from the digitally recorded micrographic images, and displayed as a distance *versus* intensity plot (**Fig 7**). The results of this semiquantitative analysis re-emphasized the similarity between C/EBPβ and CHOP in the epidermis, which appeared to rise and fall together. C/EBPα, on the other hand, followed a pattern nearly opposite to that of C/EBPβ and CHOP. This raises the interesting possibility that these factors are somehow regulated in a reciprocal manner.

As another type of measurement to confirm the specificity of the C/EBP epitopes recognized during immunohistochemical staining of the skin, extracts were prepared from mouse epidermis and analyzed by western blotting (**Fig 8**). The epidermis was separated into upper and lower fractions, using a technique that sequentially removes basal, spinous, and granular cells from the stratum corneum during vigorous agitation (see *Materials and Methods*). In our particular epidermal preparation, the split in the epidermis occurred in the mid-granular layer, an interpretation supported by the data in **Fig 8**(*A–D*) and by comparison with a large body of literature on keratin expression and modification during epidermal differentiation (Woodcock-Mitchell *et al*, 1982; Bowden *et al*, 1984). In **Fig 8**(*A*), a total protein stain revealed prominent bands in the lower fraction corresponding to the suprabasal keratins K1 (≈67 kDa) and K10 (≈59 kDa), whereas the upper fraction showed a different pattern consistent with proteolytic degradation of K1 and K10 during corneocyte formation (Bowden

et al, 1984). The lower fraction contained a significant number of basal cells, because a western blot for keratin K14 (**Fig 8C**) showed staining of a 53 kDa band that was very intense in the lower fraction, as expected (Roop *et al*, 1988). A western blot for keratin K10 (**Fig 8B**) showed approximately equal amounts of K10 in the upper and lower fractions, but with the addition of a high-molecular weight smear in the upper fraction that represented cross-linking of K10 during cornification (Schweizer and Winter, 1982). **Figure 8**(*D*), a western blot for filaggrin, further localized the apparent split to the mid-granular layer, because expression of large profilaggrin precursors (>200 kDa) is known to begin in the granular layer (as in **Fig 8D**, *lower*), with subsequent proteolytic cleavage of profilaggrin to small filaggrin units and linker peptides (**Fig 8D**, *upper*) as cells migrate into the stratum corneum (Presland *et al*, 1997). In view of the biochemical characterization of upper and lower fractions (**Fig 8A–D**), the immuno-blots for C/EBP (**Fig 8E–G**) were entirely consistent with the histochemical data shown previously in **Figs 6** and **7**. C/EBPα anti-sera (**Fig 8E**) revealed a major band at 44 kDa that was present in both upper and lower fractions. The size of the band, slightly higher than the 42 kDa band from cos cells transfected with a rat C/EBPα plasmid, could reflect species differences in the amino acid sequence (mouse *versus* rat), or differences in post-translational modification. In either case, the presence of a strong band detected by C/EBPα-specific anti-sera confirmed that the immunoreactivity observed in histologic sections represented full-length C/EBPα. For the long and short forms of CEBPβ (**Fig 8F**) and for CHOP (**Fig 8G**), bands corresponding to LAP (32 kDa), LIP (20 kDa), and CHOP (29 kDa) occurred mostly in the lower fraction as expected from their histochemical pattern within the living epidermal layers. As a final point, western analyses of basal keratinocytes collected by gentle scraping of the dermis after epidermal removal with trypsin (Marcelo *et al*, 1978) showed abundant C/EBPβ and barely detectable levels of C/EBPα and CHOP (data not shown), a pattern nearly identical to that seen in BALB/MK keratinocytes grown in low calcium (**Fig 4**).

DISCUSSION

In this study, we have shown that several members of the C/EBP transcription factor family, important regulators of differentiation-specific genes in liver and adipose tissues, are also highly expressed in the skin. In addition, our data extend recent findings of C/EBPβ expression in the nuclei of cultured human keratinocytes (Wang *et al*, 1996; Eckert *et al*, 1997) by demonstrating time-dependent changes in expression of three C/EBP isoforms in a culture model of differentiation, BALB/MK keratinocytes. These changes, along with the spatial (i.e., microanatomic) patterns of expression in the epidermis and its appendages, together support the hypothesis that one or more of the C/EBP factors play a role in epidermal differentiation.

Whereas keratinocyte culture models can only approximate the *in vivo* epidermal differentiation program (Yuspa *et al*, 1989), they have proven useful here to show that C/EBP family isoforms are expressed in keratinocytes and to identify trends in expression that may later be confirmed *in vivo*. Time-dependent increases in C/EBP are a consistent feature in other culture systems for which C/EBP have been implicated in differentiation, i.e., hepatocytes (Flodby *et al*, 1993), adipocytes (Ron *et al*, 1992), monocytes (Natsuka *et al*, 1992), lymphocytes (Saisanit and Sun, 1997), and pancreatic beta cells (Lu *et al*, 1997). The pattern of C/EBP expression in BALB/MK keratinocytes, in which levels of the full-length forms of C/EBPα and C/EBPβ (both transcriptional activators), and of CHOP (a transcriptional repressor), are low in proliferating keratinocytes, but become elevated upon raising the calcium concentration to induce differentiation, can thus be compared with the time courses in these other tissues. NIH 3T3-L1 adipoblasts provide an interesting comparison (Cao *et al*, 1991). When 3T3-L1 adipoblasts differentiate into adipocytes, increased expression of C/EBPβ occurs as an early, transient rise (days 1–3), whereas increases in C/EBPα occur later (day 5 and beyond) (Cao *et al*, 1991). In contrast, in BALB/MK cells the onsets of C/EBPα and C/EBPβ expression are not as clearly separable. It has been suggested that in the adipoblast system, in which differentiation is stimulated by the addition of
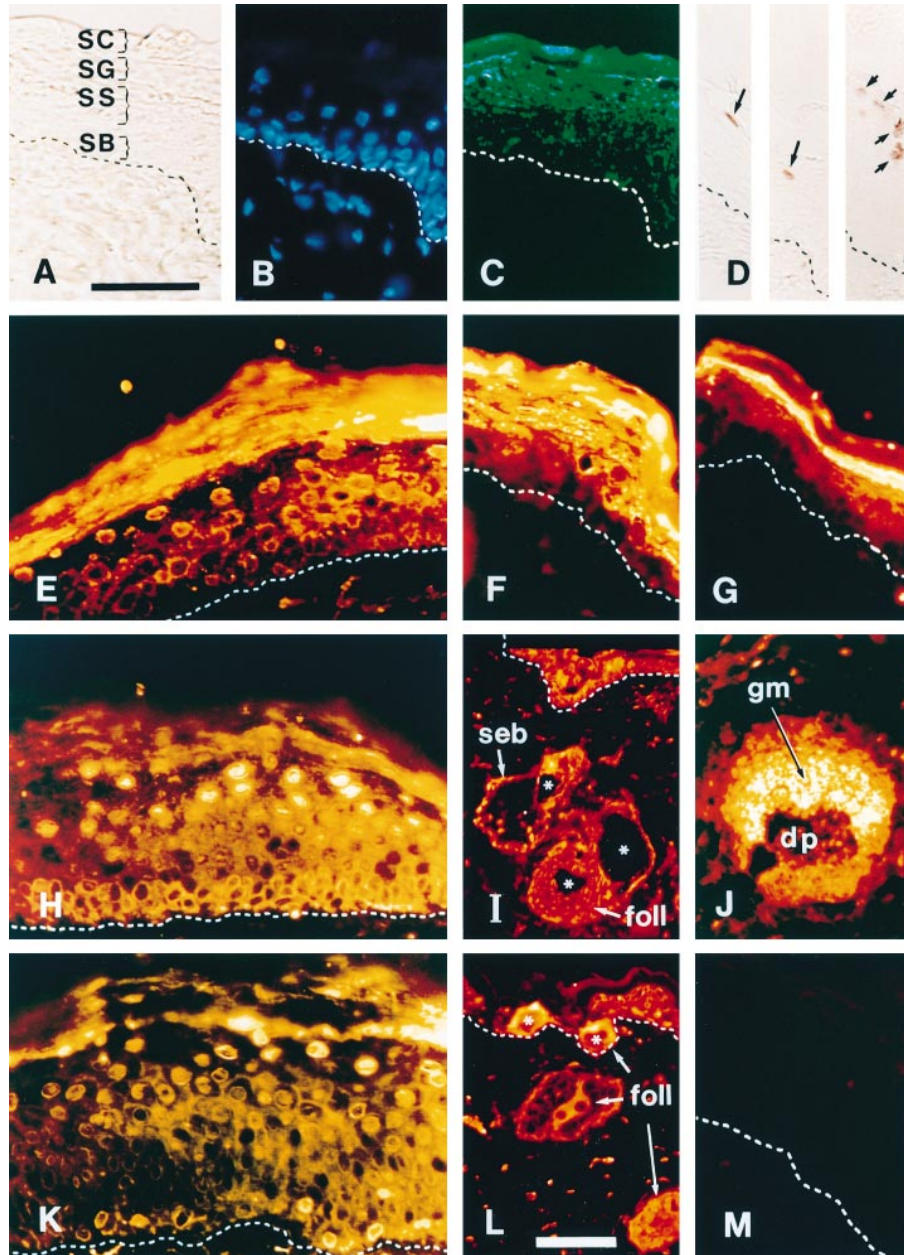
Figure 6. Immunohistochemical analysis of C/EBP in mouse, rat, and human skin. The *dotted lines* indicate the dermal–epidermal junction. Parts (*A*)–(*C*) are from the same field of rat skin, as follows: (*A*) phase-contrast (SC, stratum corneum; SG, stratum granulosum; SS, stratum spinosum; SB, stratum basale); (*B*) H33258 DNA-specific stain; (*C*) Keratin K10. (*D*) Examples of apoptotic cells (→) in the granular layer of mouse skin, as detected by TUNEL assay. The remaining parts show Cy3-labeled immunofluorescent detection of the following proteins in the skin of the designated species: (*E*) C/EBPα, human; (*F*) C/EBPα, rat; (*G*) C/EBPα, mouse; (*H*) C/EBPβ, human; (*I*) C/EBPβ, rat (seb, sebaceous gland; Foll, follicular root sheath; *, empty canals from which hair shafts have been dislodged); (*J*) C/EBPβ, in rat hair bulb (gm, germinative matrix; dp, dermal papilla); (*K*) CHOP, human; (*L*) CHOP in hair follicles, rat; (*M*) normal sera control. *Black scale bar*, 50 μm [shown in (*A*), applies to all parts except (*I*) and (*L*)]; *white scale bar*, 100 μm [shown in (*L*), applies only to (*I*) and (*L*)].

glucocorticoids plus a cAMP-elevating agent, the transient expression of C/EBPδ and C/EBPβ may correspond to early events along the differentiation pathway that are mediated by glucocorticoids and cAMP, respectively (McKnight, 1992). These steps are preparatory, allowing subsequent late expression of C/EBPα (McKnight, 1992). For keratinocytes, early events in the differentiation program are probably different to those in adipoblasts.

Apart from time-course data, the spatial distribution of C/EBP provides more evidence to support a role for these transcription factors in epidermal differentiation. Whereas the immunostaining data from the *in vitro* model are suggestive (i.e., C/EBPα, C/EBPβ, and CHOP are preferentially expressed in the squamous cells of stratifying islands), the data from the epidermis *in vivo* provide the strongest evidence for participation of C/EBP in the regulation of gene expression during epidermal differentiation. The basal layer contains abundant C/EBPβ and some CHOP, but these proteins are mostly cytoplasmic in location within basal cells; however, strong nuclear staining of C/EBPβ and CHOP develops as keratinocytes migrate upwards through the stratum spinosum. Thus, C/EBPβ and CHOP are located in the nuclei of differentiating keratinocytes *in vivo*, at a level in the epidermis where certain differentiation-specific genes are switched on. Exactly which target genes might be regulated remains unknown, but one likely candidate is keratin K10, a gene that contains at least one C/EBP consensus binding site (GCAAT) in its 5′-regulatory region (Krieg et al, 1985).

Our demonstration that C/EBP are expressed in the epidermis invites further studies on the regulation and function of these factors. Due to the ability of different C/EBP isoforms to heterodimerize with
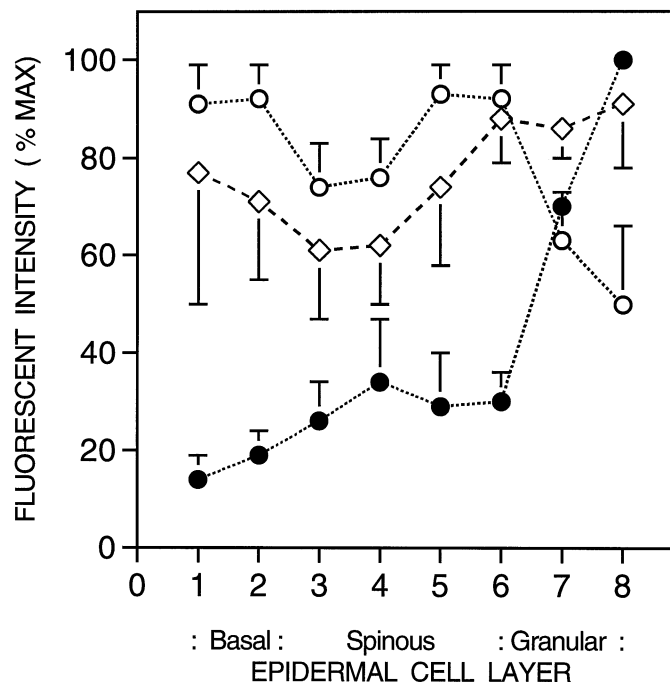
Figure 7. Quantitation of immunofluorescent staining of C/EBPα (●), C/EBPβ (○), and CHOP (◇) shows differential expression at various levels in human epidermis. Fluorescent intensity was measured at each cell layer in the epidermis, excluding the stratum corneum (see *Materials and Methods*). Data are expressed as relative fluorescent intensity (a percentage of the maximum intensity within each specimen); each point represents the mean of five images ± SD.
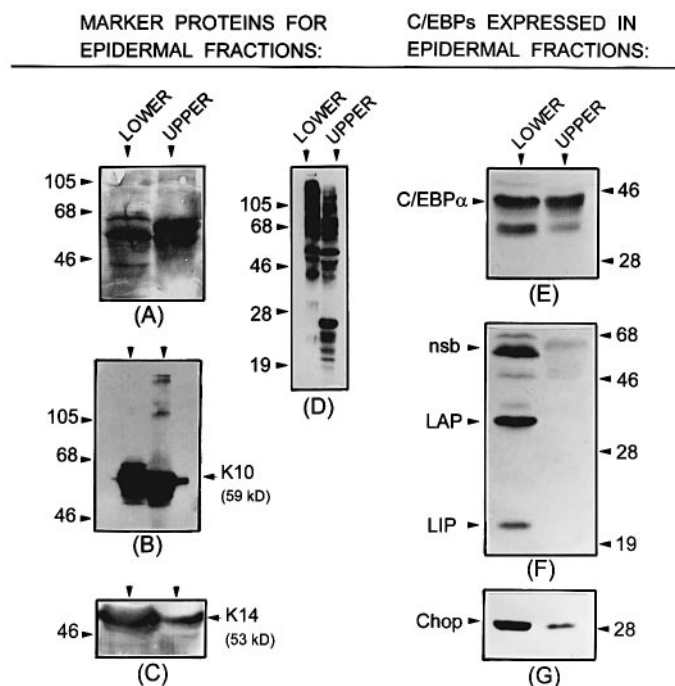

Figure 8. Western blot analyses to demonstrate expression of C/EBP in the epidermis *in vivo*. The epidermis from newborn mice was removed and separated into two protein fractions, with the lower fraction comprising nearly all of the living layers, and the upper fraction comprising the upper granular and cornified layers (see text). Equal amounts of protein were analyzed on polyacrylamide gels. Upper and lower fractions are indicated above each lane, and the positions of molecular weight markers are shown with *arrowheads* beside each blot. (*A*) Blot stained with Amido Black, a total-protein stain; (*B*) immunoblot with anti-K10 anti-serum; (*C*) immunoblot with anti-K14 serum; (*D*) immunoblot with anti-filaggrin anti-serum; (*E*) immunoblot with anti-C/EBPα anti-serum; (*F*) immunoblot with anti-C/EBPβ anti-serum. The locations of LIP and LAP are indicated by →. nsb, nonspecific band. (*G*) Immunoblot with anti-CHOP anti-serum.

one another, and the fact that some isoforms have inhibitory rather than stimulatory effects upon transcription, the details of C/EBP-mediated gene regulation in the epidermis are undoubtedly complex. For example, full-length C/EBPα or C/EBPβ are factors that usually stimulate target genes when bound as homodimers; however, the short forms of C/EBPα (C/EBP 30) and C/EBPβ (LIP), produced from internal translation start-sites within the mRNA for C/EBPα (Ossipow *et al*, 1993) and C/EBPβ (Descombes and Schibler, 1991), are known inhibitors of transcription. Their presence in keratinocytes, albeit at low levels, suggests possibilities for competition between stimulatory and inhibitory influences on transcription of certain target genes. Another known inhibitor of transcription, CHOP (Ron and Habener, 1992), is present in significant amounts in both differentiating BALB/MK keratinocytes and in the epidermis. Our observation of an apparent similarity between the expression patterns of CHOP and C/EBPβ in the epidermis, whereby the levels of the two proteins appear to parallel one another (**Figs 6, 7**), is intriguing. Given the current understanding that C/EBPβ is an activator and CHOP is a repressor, one might hypothesize that CHOP and C/EBPβ expression are linked because CHOP inhibits C/EBPβ stimulation, preventing an overshoot of target gene upregulation.

Some of our observations pose questions that are not readily answerable. For example, does the predominantly cytoplasmic location of C/EBPα serve to preclude a role in transcriptional regulation? Could C/EBPα regulate transcription indirectly, e.g., by sequestering other related proteins, such as activator forms of C/EBPβ or repressors such as CHOP? These and other questions must await further studies.

REFERENCES

An MR, Hsieh C-C, Reisner PD, Rabek JP, Scott SG, Kuninger DT, Papaconstantinou J: Evidence for posttranscriptional regulation of C/EBPα and C/EBPβ isoform expression during the lippolysaccharide-mediated acute-phase response. *Mol Cell Biol* 16:2295–2306, 1996

Ausbel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K: *Current Protocols in Molecular Biology*. John Wiley, New York, 1992, p. 9.7

Birkenmeier EH, Gwynn B, Howard S, Jerry J, Gordon JI, Landschulz WH, McKnight SL: Tissue-specific expression, developmental regulation, and genetic mapping of the gene encoding CCAAT/enhancer binding protein. *Genes Dev* 3:1146–1156, 1989

Bowden PE, Quinlan RA, Breitzreutz D, Fusenig NE: Proteolytic modification of acidic and basic keratins during terminal differentiation of mouse and human epidermis. *Eur J Biochem* 142:29–36, 1984

Cao Z, Umek RM, McKnight SL: Regulated expression of three C/EBP isoforms during adipose conversion of 3T3–L1 cells. *Genes Dev* 5:1538–1552, 1991

Collier NC, Schlesinger MJ: The dynamic state of heat shock proteins in chicken embryo fibroblasts. *J Cell Biol* 103:1495–1507, 1986

Costa RH, Grayson DR, Xanthopoulos KG, Darnell JE Jr A liver-specific DNA-binding protein recognizes multiple nucleotide sites in regulatory regions of transthyretin, a–1-antitrypsin, albumin, and SV40 genes. *Proc Natl Acad Sci USA* 85:3840–3844, 1988

Descombes P, Schibler U: A liver-enriched transcriptional activator protein, LAP, and a transcriptional inhibitory protein, LIP, are translated from the same mRNA. *Cell* 67:569–579, 1991

Eckert RL, Crish JF, Banks EB, Welter JF: The epidermis: Genes on – genes off. *J Invest Dermatol* 109:501–509, 1997

Flodby P, Antonson P, Barlow C, Blanck A, Porsch-Hallstrom I, Xanthopoulos KG: Differential patterns of expression of three C/EBP isoforms, HNF-1, and HNF-4 after partial hepatectomy in rats. *Exper Cell Res* 208:248–256, 1993

Freytag SO, Paielli DL, Gilbert JD: Ectopic expression of the CCAAT/enhancer-binding protein α promotes the adipogenic program in a variety of mouse fibroblastic cells. *Genes Dev* 8:1654–1663, 1994

Friedman AD, Landschulz WH, McKnight SL: CCAAT/enhancer binding protein activates the promoter of the serum alumin gene in cultured hepatoma cells. *Genes Dev* 3:1314–1322, 1989

Fuchs E: Epidermal differentiation: the bare essentials. *J Cell Biol* 111:2807–2814, 1990

Graves BJ, Johnson PF, McKnight SL: Homologous recognition of a promoter domain common to the MSV LTR and the HSV tk gene. *Cell* 44:565–576, 1986

Haas NB, Cantwell CA, Johnson PF, Burch JBE: DNA-binding specificity of the PAR basic leucine zipper protein VBP partially overlaps those of the C/EBP and CREB/ATF families and is influenced by domains that flank the core basic region. Mol Cell Biol 15:1923–1932, 1995

Hennings H, Michael D, Cheng C, Steinert P, Holbrook K, Yuspa SH: Calcium regulation of growth and differentiation of mouse epidermal cells in culture. Cell 19:245–254, 1980

Krieg TM, Schafer MP, Cheng CK, Filpula D, Flaherty P, Steinert PM, Roop DR: Organization of a type I keratin gene: evidence for evolution of intermediate filaments from a common ancestral gene. J Biol Chem 260:5867–5870, 1985

Lamb P, McKnight SL: Diversity and specificity in transcripional regulation: the benefits of heterotypic dimerization. Trends Biochem Sci 16:417–422, 1991

Landschulz WH, Johnson PF, Adashi EY, Graves BJ, McKnight SL: Isolation of a recombinant copy of the gene encoding C/EBP. Genes Dev 2:786–800, 1988

Lin F-T, Lane MD: Antisense CCAAT/enhancer-binding protein RNA suppresses coordinate gene expression and triglyceride accumulation during differentiation of 3T3–L1 preadipocytes. Genes Dev 6:533–544, 1992

Lin F-T, Lane MD: CCAAT/enhancer binding protein α is sufficient to initiate the 3T3–L1 adipocyte differentiation program. Proc Natl Acad Sci USA 91:8757–8761, 1994

Lu M, Seufert J, Habener JF: Pancreatic β-cell specific repression of insulin gene transcription by CCAAT/Enhancer-binding protein β. J Biol Chem 272:28349–28359, 1997

Luethy JD, Fargnoli J, Park JS, Fornace AJ, Holbrook NJ: Isolation and characterization of the hamster gadd153 gene: activation of promoter activity by agents that damage DNA. J Biol Chem 265:16521–16526, 1990

Marcelo CL, Kim YG, Kaine JL, Voorhees JJ: Stratification, specialization, and proliferation of primary keratinocyte cultures. J Cell Biol 79:356–370, 1978

Maytin E: Differential effects of heat shock and UVB light upon stress protein expression in epidermal keratinocytes. J Biol Chem 267:23189–23196, 1992

Maytin EV, Murphy LA, Merrill MA: Hyperthermia induces resistance to ultraviolet light B in primary and immortalized epidermal keratinocytes. Cancer Res 53:4952–4959, 1993

Maytin EV, Wimberly JM, Kane KS: Heat shock modulates UVB–induced apoptosis in human epithelial keratinocytes: evidence for a hyperthermia inducible protective response. J Invest Dermatol 103:547–553, 1994

McKnight SL: CCAAT/Enhancer binding protein. In: McKnight SL, Yamamoto KR (eds). Transcriptional Regulation. Cold Spring Harbor Laboratory Press, New York, 1992, pp. 771–795

Metz R, Ziff E: cAMP stimulates the C/EBP-related transcription factor rNFIL-6 to translocate to the nucleus and induce c-fos transcription. Genes Dev 5:1754–1766, 1991

Natsuka S, Akira S, Nishio Y, Hashimoto S, Sugita T, Isshiki H, Kishimoto T: Macrophage differentiation-specific expression of NF-IL6, a transcription factor for interleukin-6. Blood 79:460–466, 1992

Ossipow V, Descombes P, Schibler U: CCAAT/enhancer-binding protein mRNA is translated into multiple proteins with different transcription activation potentials. Proc Natl Acad Sci USA 90:8219–8223, 1993

Presland RB, Kimball JR, Kautsky MB, Lewis SP, Lo CY, Dale BA: Evidence for specific proteolytic cleavage of the N-terminal domain of human profilaggrin during epidermal differentiation. J Invest Dermatol 108:170–178, 1997

Ron D, Habener JF: CHOP, a novel developmentally regulated nuclear protein that dimerizes with transcription factors C/EBP and LAP and functions as a dominant-negative inhibitor of gene transcription. Genes Dev 6:439–453, 1992

Ron D, Brasier AR, McGehee REJ, Habener JF: Tumor necrosis factor-induced reversal of adipocytic phenotype of 3T3–L1 cells is preceded by a loss of nuclear CCAAT/Enhancer binding protein (C/EBP). J Clin Invest 89:223–233, 1992

Roop DR, Krieg TM, Mehrel T, Cheng CK, Yuspa SH: Transcriptional control of high molecular weight keratin gene expression in multistage mouse skin carcinogenesis. Cancer Res 48:3245–3252, 1988

Rorth P, Montell DJ: Drosophila C/EBP: a tissue-specific DNA-binding protein required for embryonic development. Genes Dev 6:2299–2311, 1992

Saisanit S, Sun X-H: Regulation of the pro B-cell-specific enhancer of the Id1 gene involves the C/EBP family of proteins. Mol Cell Biol 17:844–850, 1997

Schweizer J, Winter H: Keratin polypeptide analysis in fetal and in terminally differentiating newborn mouse epidermis. Differentiation 22:19–24, 1982

Simonson MS, LePage DF, Walsh K: Rapid characterization of growth-arrest genes in transient transfection assays. Biotechniques 18:434–442, 1995

Sportsman JR, Elder JH: A microanalytical protein assay using laser densitometry. Anal Biochem 139:298–300, 1984

Trautwein C, Caelles C, van der Geer P, Hunter T, Karin M, Chojkier M: Transactivation by NF-IL/LAP is enhanced by phosphorylation of its activation domain. Nature 364:544–547, 1993

Ubeda M, Wang X–Z, Zinszner H, Wu I, Habener JF, Ron D: Stress-induced binding of the transcription factor CHOP to a novel DNA control element. Mol Cell Biol 16:1479–1489, 1996

Vinson CR, Hai T, Boyd SM: Dimerization specificity of the leucine zipper-containing bZIP motif on DNA binding: prediction and rational design. Genes Dev 7:1047–1058, 1993

Wang H, Liu K, Yuan F, Berdichevsky L, Taichman LB: C/EBPbeta is a negative regulator of human papillomavirus type 11 in keratinocytes. J Virol 70:4839–4844, 1996

Wang XZ, Ron D: Stress-induced phosphorylation and activation of the transcription factor CHOP (gadd 153) by p38 MAP kinase. Sci 272:1347–1349, 1996

Weissman B, Aaronson S: BALB and Kirsten murine sarcoma viruses alter growth and differentiation of EGF-dependent BALB/c mouse epidermal keratinocyte lines. Cell 32:599–606, 1983

Woodcock-Mitchell J, Eichner R, Nelson WG, Sun TT: Immunolocalization of keratin polypeptides in human epidermis using monclonal antibodies. J Cell Biol 95:580–588, 1982

Young DA, Voris BP, Maytin EV, Colbert RA: Very-high-resolution two-dimensional electrophoretic separation of proteins on giant gels. Meth Enzymol 91:190–214, 1983

Yuspa S, Dlugosz AA: Cutaneous carcinogenesis: natural and experimental. In: Goldsmith LA (ed.). Physiology, Biochemistry, and Molecular Biology of the Skin, 2nd edn. Oxford University Press, New York, 1991, pp. 1365–1402

Yuspa S, Hennings H, Tucker R, Jaken S, Kilkenny A, Roop D: Signal transduction for proliferation and differentiation in keratinocytes. Ann NY Acad Sci 548:191–196, 1988

Yuspa S, Kilkenny AE, Steinert PM, Roop DR: Expression of murine epidermal differentiation markers is tightly regulated by restricted extracellular calcium concentrations in vitro. J Cell Biol 109:1207–1217, 1989