The roles of peroxisome proliferator-activated receptors (PPARs) and CCAAT/enhancer-binding proteins (C/EBPs) in keratinocyte and sebocyte differentiation suggest that both families of transcription factors closely interact in the skin. Initial characterization of the mouse PPARβ promoter revealed an AP-1 site that is crucial for the regulation of PPARβ expression in response to inflammatory cytokines in the skin. We now present evidence for a novel regulatory mechanism of the expression of the PPARβ gene by which two members of the C/EBP family of transcription factors inhibit its basal promoter activity in mouse keratinocytes. We first demonstrate that C/EBPα and C/EBPβ, but not C/EBPδ, inhibit the expression of PPARβ by the recruitment of a transcriptional repressor complex containing HDAC-1 to a specific C/EBP binding site on the PPARβ promoter. Consistent with this repression, the expression patterns of PPARβ and C/EBPs are mutually exclusive in keratinocytes of the interfollicular epidermis and hair follicles in mouse developing skin. This work reveals the importance of the regulatory interplay between PPARβ and C/EBP transcription factors in the control of proliferation and differentiation in this organ. Such insights are crucial for the understanding of the molecular control regulating the balance between proliferation and differentiation in many cell types including keratinocytes.

Peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcription factors that belong to the nuclear hormone receptor family. Three isoforms encoded by separate genes have been identified in vertebrates, PPARα (NR1C1), PPARβ/δ (NR1C2; called β below), and PPARγ (NR1C3), which have a variety of functions (1). Most particularly, the importance of PPARs in regulating lipid metabolism (2) has led to investigate PPAR expression and function during the differentiation of the skin, a tissue with high rates of fatty acid and cholesterol metabolism. We previously showed that C/EBPα and C/EBPβ, are expressed in the interfollicular epidermis and hair follicles during embryonic mouse development (3).

In adult skin, PPARβ is very low in interfollicular keratinocytes, but its expression is reactivated upon proliferative stimuli such as cutaneous injury and hair plucking (4). Using specific PPAR agonists and in vivo gene disruption approaches in mice, we demonstrated the critical role of PPARβ in regulating the balance between proliferation and apoptosis in keratinocytes during skin wound healing (4, 5), as well as during postnatal hair follicle development (6), through direct activation of the anti-apoptotic phosphatidylinositol 3-kinase/Akt1 signaling pathway (7, 8). Furthermore, PPARβ was reported to keratinocyte differentiation and epidermal permeability maturation under normal and inflammatory conditions (3, 5).

CCAAT/enhancer-binding proteins (C/EBPs) are members of the basic leucine zipper family of transcription factors and also play pivotal roles in the regulation of human and mouse skin homeostasis. Genes coding for six C/EBP isoforms (C/EBPα, C/EBPβ, C/EBPδ, C/EBPγ, C/EBPε, and C/EBPζ or CHOP-10) have been cloned and characterized in mammalian cells (9). All C/EBPs consist of three structural domains: a C-terminal leucine zipper domain, a highly conserved canonical basic region, and an N-terminal domain, which contains both positive and negative regulatory regions (10). The basic region allows binding to specific palindromic CCAAT motifs located in the promoter of C/EBP target genes (11), whereas the leucine zipper motif is responsible for homo- and heterodimerization between C/EBP members, which is absolutely required for DNA binding as well as transcriptional activity. C/EBPs also interact with many other basic leucine zipper and non-basic leucine zipper factors such as NF-kB, p21, and activator protein-1 (AP-1) (9). They control the transcription of many key genes, either as transcriptional activators or repressors, demonstrating the importance of this family of transcription factors in the regulation of a number of cellular processes, including energy metabolism, inflammation, and liver regeneration (9, 10). In addition, they are well known regulators of the balance between differentiation and proliferation in various cell types like adipocytes, keratinocytes, granulocytes, and hepatocytes (9, 12). In the skin, C/EBPα and C/EBPβ are the most abundantly expressed isoforms in keratinocytes from the interfollicular and follicular epidermis (13–16). Proliferative and antiproliferative functions of these two proteins have been characterized in keratinocytes, on the basis of their expression profiles and presence of binding sites in the promoter region of genes encoding early and late keratinocyte differentiation markers, such as keratin 10 (K10), K1, and involucrin (13–19). In accordance, analysis of C/EBPβ-null epidermis revealed a mild epidermal hyperplasia and slightly decreased expression of epidermal differentiation markers (15). Finally, it has been recently reported that C/EBPα and C/EBPβ have a specific expression pattern during the mouse hair growth cycle, suggesting that C/EBP family members may also regulate gene expression during hair cycling (16) and sebocyte differentiation (20).
The important roles of PPARs and C/EBPs identified in skin strongly suggest that both families of transcription factors may closely interact to regulate the epidermal and/or hair follicle differentiation program. In favor of this hypothesis, an interplay between PPARs and C/EBPs was observed during the differentiation of adipocytes (21), sebocytes (20), and astrocytes (22). In this study, we provide compelling evidence for the transcriptional repression in mouse keratinocytes of PPARβ expression by C/EBPα and C/EBPβ, through a mechanism that requires both binding to DNA and histone deacetylation. Consistent with this, PPARβ and C/EBP expression are mutually exclusive in the interfollicular epidermis and hair follicles of mouse skin. Such interplay between PPARβ and C/EBP transcription factors is crucial in the molecular control of the balance between differentiation and proliferation in keratinocytes and many other cell types.

**EXPERIMENTAL PROCEDURES**

**Reagents**—The histone deacetylase inhibitor trichostatin A was from Cell Signaling (catalog number 9950). All cell culture media and supplies were obtained from Sigma. The following antibodies were used: anti-PPARβ (PA1–823), anti-HDAC-1 (PA1–860), and anti-HDAC-2 (PA1–861) from Affinity Bioreagents; anti-acetyl-histone H4 (catalog number 06-866) from Upstate Biotechnology, Inc. (Lake Placid, NY); anti-C/EBPα (sc-61), anti-C/EBPβ (sc-150), anti-HDAC-1 C-19 (sc-6298), HA-probe (sc-805), and fluorescein isothiocyanate-conjugated secondary antibodies (sc-2012) from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); anti-HA tag (catalog number 2362) from Cell Signaling; and anti-β-tubulin (catalog number 565621) from PharMingen.

**Plasmid Constructs**—cDNAs encoding mouse C/EBPα (m/C/EBPα), m/C/EBPβ and m/C/EBPβ were subcloned with an N-terminal epitope tag into pCMV-HA (Clontech). m/C/EBPα and m/C/EBPβ deletion mutants were generated using specific PCR amplification according to the arrangement of their structural domains (10). The dominant negative m/C/EBPα (K299E) and m/C/EBPβ (K238E) were created by using the QuikChange™ site-directed mutagenesis kit from Stratagene (23). The proximal 2-kb mouse cyclooxygenase-2 promoter (6) and 0.2-kb mouse K10 promoter (13) regions were subcloned into XhoI/HindIII sites of the promoterless pGL2 luciferase vector (Promega). The full-length (1880 bp, accession number AF329818) and truncated proximal mouse PPARβ promoter regions were previously described (5) and were subcloned upstream of a promoterless luciferase reporter construct (pGL-luc) (24). Site-directed mutagenesis of the C/EBP binding site at position −494/−495 into the PPARβ promoter was achieved using the QuikChange™ mutagenesis kit.

**Cell Culture and Transient Transfections**—Mouse BALB/MK keratinocytes were grown in Eagle’s minimum essential medium (Joklik modification) containing 10% dialyzed fetal calf serum, 0.05 mM CaCl₂, 10 ng/ml epidermal growth factor, and 5 μg/ml gentamycin. Transient transfection assays were performed in 12-well plates using Superfect reagent (Qiagen), and luciferase activity was measured with the Promega dual reporter kit, according to the manufacturer’s instructions. To reduce the background, all transfections were performed in the absence of fetal calf serum.

**Real Time PCR**—Total RNA from mouse keratinocytes was isolated using TRIzol reagent (Invitrogen). cDNA was generated by reverse transcription using 1 μg of total RNA (GeneAmp Gold RNA PCR reagent kit; Applied Biosystems) and analyzed by quantitative PCR using the SYBR Green I kit (Eurogentec) and the ABI Prism 7700 sequence detector. The thermocycler was programmed as follows: 95 °C for 10 min; 45 cycles of 95 °C for 15 s; 60 °C for 1 min. The housekeeping gene hypoxanthine phosphoribosyltransferase was used for normalization. The following primers were used: forward primer PPARβ, 5’-CGGCGACGCTTCAACATGG-3’; reverse primer PPARβ, 5’-AGATCCGATCCACCTTCTTACAT-3’; forward primer PPARγ, 5’-TGATTACAAATATGATCGTGAAGCTCC-3’; reverse primer PPARγ, 5’-TTGTAGACGGGTCTTTTCAGAAT-3’; forward primer hypoxanthine phosphoribosyltransferase, 5’-TTAAGCAGTACGCCCCAAATG-3’; reverse primer hypoxanthine phosphoribosyltransferase, 5’-TCTTTTTCACCAGCAACGTTG-3’.

**In Vivo Protein-Protein Cross-link and Chromatin Immunoprecipitation (ChIP)**—Protein-protein cross-link and ChIP were performed as previously described (8) with some modifications. Cells were fixed with 1% formaldehyde at 37 °C for 15 min before sonication in Iysis buffer (10 mM EDTA, 1% SDS, 50 mM Tris·HCl, pH 8.1, protease inhibitor mixture (Roche Applied Science)) to obtain cross-linked DNA fragments of 200–600 bp in length. The immunoprecipitates were reverse cross-linked for PCR or boiled for 5 min in SDS loading buffer for Western blot analysis. For double chromatin immunoprecipitation (re-ChIP) assays, complexes were eluted from the agarose beads by sequential incubation with one volume of Immunopure Gentle Ag/Ab elution buffer (Pierce), followed by one volume of the same buffer containing 0.1 mM dithiothreitol. The eluates were pooled, diluted 20-fold in re-ChIP dilution buffer (1 mM EDTA, 50 mM NaCl, 1% Triton X-100, 20 mM Tris·HCl, pH 8.1), and subjected to another ChIP procedure. ChIP and re-ChIP assays were done using anti-acetyl-histone H4, HA-probe, and/or HDAC-1 C-19 antibodies. PCR was performed using 25 cycles with primers flanking either the C/EBP response element (−567 to −284) or an unrelated control sequence (−1179 to −894) on the mouse PPARβ promoter.

**Immunohistochemistry**—Immunofluorescent staining on mouse skin cryosections was carried out as follows: fixation for 5 min with 75% acetone and 25% ethanol; blockage for 1 h with 5% normal goat serum and 3% bovine serum albumin; incubation with the primary antibody (C/EBPα, 1:100; C/EBPβ, 1:100) for 1 h with 5% normal goat serum; incubation with the fluorescein isothiocyanate-conjugated secondary antibody (dilution of 1:200) for 1 h with 5% normal goat serum. Samples were mounted in 4’,6’-diamidino-2-phenylindole-containing Vectashield mounting medium (Vector Laboratories). Colorimetric staining for PPARβ was carried out as previously described (6).

**Western Blot Assays**—Western blots were performed according to standard procedures, as previously described (6). Primary antibodies against HDAC-1, HDAC-2, and β-tubulin were used at dilutions of 1:2000, and the anti-HA antibody was diluted 1:1000. Detection was performed using chemiluminescence (Pierce) with horseradish peroxidase.

**RESULTS**

PPARβ, C/EBPα, and C/EBPβ Are Highly Expressed in the Developing Mouse Skin—The important roles of PPAR and C/EBP transcription factors in regulating overlapping pathways in keratinocytes suggest that both families of transcription factors closely interact to regulate the differentiation program of the skin. In favor of this hypothesis, similar expression patterns of PPARs and C/EBPs were previously reported during sebocyte differentiation in this organ (20). Therefore, we first performed a detailed analysis of the expression of PPARβ and of the two main C/EBP isoforms, C/EBPα and C/EBPβ, in the developing mouse skin from postnatal day 1 to 7 (P1–P7) by immunohistochemistry (Fig. 1). As we previously described (6), the PPARβ protein is highly expressed in the basal layer keratinocytes of the interfollicular epidermis as well as in the proliferative epithelial compartments of developing hair follicles, including hair pegs and hair matrix (Fig. 1A). On the contrary,
Interplay between PPARβ/δ and C/EBPs in Keratinocytes

**FIGURE 1. Expression of PPARβ and C/EBPs in the developing mouse skin.** A and B, cryosections of mouse dorsal skin from postnatal days P1, P4, and P7 were processed either for the detection of PPARβ by colorimetric immunostaining (A) or for C/EBPα and C/EBPβ by immunofluorescence (green signal) (B). PPARβ, C/EBPα, and C/EBPβ are highly expressed in the interfollicular epidermis (EP) and sebaceous gland (SG) cells of hair follicles. In the developing hair follicles, specific expression of C/EBPα was observed in the dermal papilla fibroblasts (DP), whereas C/EBPβ was mainly found in the outer root sheath (ORS) cells as well as in the precortex region (P4). In contrast to PPARβ expression, no C/EBP staining was observed in hair peg (HP) and hair matrix (HM) keratinocytes. Cell nuclei were counterstained with 4',6-diamidino-2-phenylindole (blue). The epidermal-dermal junction is indicated by dashed lines. Magnification bars, 50 μm.

and consistent with previous reports (13, 14), C/EBPα is mainly expressed in the nuclei of keratinocytes located in all of the suprabasal layers of the interfollicular epidermis, whereas C/EBPβ is found in low amounts in some basal keratinocytes and strongly increases in the lower suprabasal layers (Fig. 1B; P1). In hair follicles, PPARβ and C/EBPs strictly colocalize in the developing sebaceous glands (Fig. 1; P4–P7), consistent with previous observations made in adult skin (16) and cultured sebocytes (20). Intense C/EBPα expression was also specifically detected in the nuclei of dermal papilla fibroblasts, from early hair follicle stages (P1) to mature follicles (P7), whereas C/EBPβ was found restricted to the outer root sheath keratinocytes. A weak staining of C/EBPβ was also observed in hair shaft precursors (precortex region) of differentiating hair follicles (Fig. 1B; P7). However, in contrast to PPARβ expression, neither C/EBPα nor C/EBPβ expression was detectable in the highly proliferative epithelial compartments of developing hair follicles, including hair peg (P1) and hair matrix (P4–P7) keratinocytes.

Altogether, these results indicate that the expression of PPARβ and C/EBPs is mutually exclusive in mouse keratinocytes in the interfollicular epidermis as well as in the differentiating hair follicles, suggesting a negative interplay in keratinocytes between the two families of transcription factors during skin development.

**C/EBPs Modulate PPARβ Expression in Mouse Keratinocytes**—To assess whether C/EBPs could directly modulate PPARβ expression in keratinocytes, mouse BALB/MK cells were transiently co-transfected with C/EBP-expressing vectors and a luciferase reporter construct containing the proximal promoter of the mouse PPARβ gene. This 1.88-kb PPARβ promoter region used herein was characterized in our laboratory. It contains sequences related to binding sites for AP-1, Ets, and C/EBP transcription factors (5).

Overexpression in BALB/MK cells of either C/EBPα or C/EBPβ repressed basal PPARβ promoter activity by 2–3-fold in a dose-dependent manner, whereas expression of C/EBPδ had no significant effect (Fig. 2A). Consistent with these observations, transfection of C/EBPα and C/EBPβ in mouse keratinocytes resulted in a decrease of endogenous PPARβ mRNA levels, as evaluated by real time PCR (Fig. 2B, left). In similar conditions, PPARγ expression was increased, with C/EBPα and C/EBPδ having a greater effect than C/EBPβ (Fig. 2B, right). As a control of the functionality of the transfected C/EBPs, we used the proximal murine promoter regions of the genes for keratin 10 (K10) and cyclooxygenase-2, which both contain multiple functional C/EBP-binding sites (13, 25). As previously described in other cell types, a strong activation of the K10 promoter by both C/EBPα and C/EBPβ was observed in BALB/MK cells (Fig. 2C, left) (13), whereas the cyclooxygenase-2 promoter activity, in agreement with a previous report, was only increased by C/EBPδ (Fig. 2C, right) (25).

To further identify the region in the mouse PPARβ promoter that mediates the inhibition described above, the consequence of C/EBPα and C/EBPβ overexpression on the activity of a series of truncations of...
the proximal PPARβ promoter was analyzed in BALB/MK cells. As shown in Fig. 3A, C/EBPα and C/EBPβ still inhibited the activity of the promoter constructs PPARβ (−846) and PPARβ (−587). In contrast, the shorter promoter constructs PPARβ (−445) and PPARβ (−223) were not responsive to C/EBPα or C/EBPβ overexpression, suggesting that the region mediating inhibition by C/EBPs is located between nucleotides −587 and −445 of the PPARβ promoter. Sequence analysis of this region revealed the presence of a putative C/EBP response element between nucleotides −494 and −485, as well as putative binding sites for Oct1 and AP-1 transcription factors (Fig. 3B). To determine whether C/EBP-mediated inhibition of PPARβ expression requires this newly identified C/EBP response element, we introduced mutations known to prevent recognition by C/EBP transcription factors (Fig. 3B) (11). Interestingly, disruption of the putative C/EBP response element in the PPARβ (−587) reporter construct totally abrogated the inhibitory effect of both C/EBPα and C/EBPβ (Fig. 3C), suggesting that the action of these factors on the activity of the PPARβ promoter depends on the occupancy of the C/EBP response element at position −494/−495.

C/EBPα and C/EBPβ Inhibit PPARβ Promoter Activity Independently of Their Repression Domains—C/EBPα and C/EBPβ are known to inhibit the promoter activity of a number of genes in various cell types, although the molecular mechanisms of this repression have not been explored (26–28). Intrinsic domains that negatively regulate transcriptional activity have been identified in C/EBPs (10), and the inhibitory functions of these domains have been attributed to a repression of DNA binding, transcriptional activation, and synergy with factors bound to adjacent promoter elements.

To determine the functional domains of C/EBPα and C/EBPβ required for inhibition of the PPARβ promoter activity, several C/EBP mutant proteins tagged with hemagglutinin (HA) were transiently overexpressed in BALB/MK keratinocytes (Fig. 4A). These mutants were deleted of the repression domain and/or of the activation domains or carried single point mutations (KE) in the DNA-binding basic region (23) that prevent binding of C/EBPs to DNA, as confirmed using ChIP assays on the K10 promoter (Fig. S1A). All C/EBP mutants were expressed at comparable protein levels when transiently transfected in mouse keratinocytes (Fig. 4B). As expected, they all have lost their ability to transactivate the K10 promoter (supplemental Fig. S1B), which requires both binding to specific DNA response elements and transactivation activity (13). Interestingly, deletion that includes the repression domain of C/EBPα (C/EBPα-A1) did not prevent its ability to inhibit PPARβ promoter activity (Fig. 4A), suggesting that C/EBPα uses an alternative mechanism to repress PPARβ transcription. Most importantly, further deletion of the region up to amino acids 170–273 in the C/EBPα protein (C/EBPα-Δ2) or deletion of the corresponding region
on C/EBPβ protein (amino acids 115–211, C/EBPβΔ2) abrogated the inhibitory effects of C/EBPs on the PPARβ promoter (Fig. 4A). Similar results were obtained by mutating the basic domain of C/EBPα (C/EBPα-KE) and C/EBPβ (C/EBPβ-KE), suggesting that the inhibitory effect of C/EBPs on PPARβ promoter requires both an internal domain spanning residues 170–273 for C/EBPα or residues 115–210 for C/EBPβ as well as an intact DNA binding domain. To confirm that the inhibitory effect of C/EBPα and C/EBPβ on the PPARβ promoter requires binding to DNA in vivo, chromatin immunoprecipitation assays were performed in BALB/MK cells transfected with the HA-tagged C/EBPα and C/EBPβ mutants. DNA fragments encompassing the C/EBP response element or a random control sequence of the endogenous mouse PPARβ promoter were amplified by PCR in the immunoprecipitated chromatin with anti-HA antibodies. As shown in Fig. 5, the sequence spanning the C/EBP response element was significantly enriched compared with the control DNA fragment for all C/EBP mutants, except for the proteins with mutated DNA binding domain (KE). Together, these results demonstrate that C/EBPα and C/EBPβ bind to the C/EBP response element identified at position −494/−495 in the PPARβ promoter and indicate that the internal domain of C/EBPs needed to repress the promoter activity of PPARβ (Fig. 4A) is not required for DNA binding.

C/EBP-mediated Inhibition of PPARβ Promoter Activity Involves Histone Deacetylation—Since histone acetylation/deacetylation is a key component in the regulation of gene expression, we next assessed the importance of this modification in the repression activity of C/EBPs on the PPARβ promoter. Transient transfections were performed in the presence of the most potent mammalian histone deacetylase inhibitor trichostatin A (29). As shown in Fig. 6A, the inhibitory effect of C/EBPα and C/EBPβ on PPARβ promoter activity was largely abrogated in the presence of trichostatin A in a dose-dependent manner, suggesting that transcriptional repression by C/EBPs occurs via promoting histone deacetylation on the PPARβ promoter. In favor of this model, C/EBPβ was recently reported to repress gene transcription in preadipocytes.
Through its direct association with a general transcription corepressor complex containing histone deacetylase-1 (HDAC-1) (30). To test whether C/EBPs and HDACs similarly interact with each other in keratinocytes in vivo, BALB/MK cells were transfected with the HA-tagged C/EBP mutants described above, and immunoprecipitations were carried out on cell extracts using anti-HA antibodies. The presence of class I HDACs interacting with the C/EBPs was detected by Western blot analysis (Fig. 6B). The results indicate that full-length C/EBPα and C/EBPβ strongly interact with HDAC-1, but not HDAC-2, in mouse keratinocytes. Most importantly, C/EBPα-Δ2 and C/EBPβ-Δ2 mutants, which failed to inhibit PPARβ promoter activity in transfaction assays (Fig. 4A), also lost their interaction with HDAC-1, suggesting again that...
C/EBP-mediated inhibition of PPARβ expression most probably involves histone deacetylation.

We next examined whether histone H4 is acetylated at the endogenous PPARβ promoter in keratinocytes transfected with the C/EBP mutants, using chromatin immunoprecipitation assays with acetyl-H4 antibodies. H4 acetylation was diminished in cells transfected with full-length C/EBPα and C/EBPβ, with C/EBPα having a greater effect than C/EBPβ, confirming that C/EBPs induce histone deacetylation, therefore decreasing PPARβ promoter activity (Fig. 7A). Most importantly, C/EBP mutants that lost their capacity to bind DNA (C/EBP-KE) or to interact with HDAC-1 (C/EBP-Δ2) also failed to decrease H4 acetylation. These results are consistent with transient transfection assays showing that the overexpression of these two mutants did not affect the basal PPARβ promoter activity (Fig. 4) and with the lack of interaction of this mutant with HDAC-1 (Fig. 6). To confirm that C/EBPs inhibit PPARβ promoter activity via recruitment of HDAC-1, we next performed two ChIP experiments (ChIP/re-ChIP) in mouse keratinocytes transfected with C/EBPα or C/EBPβ. As shown in Fig. 7B, PCR amplification of the PPARβ promoter region encompassing the C/EBP response element was observed following sequential

---

**FIGURE 5. Binding of C/EBPα and C/EBPβ to the C/EBP response element at position –494/-485 on the PPARβ promoter.** A and B, schematic representations of the mouse PPARβ promoter region with the C/EBP response element (position –494/-485) are illustrated (top). The bars below each construct represent the relative positions and the length of the fragments that are amplified by PCR. DNA-protein complexes from BALB/MK cells transfected with vectors expressing or not expressing (ctr) the indicated C/EBP entire protein or mutants were immunoprecipitated (ChIP) with either an anti-HA tag antibody (anti-HA) or control immunoglobulins (control IgG). Enrichment of either DNA fragment encompassing the C/EBP response element (A) or a control DNA sequence (B) on the endogenous mouse PPARβ promoter was evaluated by PCR. Aliquots of the chromatin were also analyzed before immunoprecipitation (input). One representative result of three independent experiments is shown.
immunoprecipitation with anti-HA and anti-HDAC-1 antibodies. No amplification was observed after a second immunoprecipitation with IgG immunoglobulins. Altogether, these results clearly demonstrate the formation of a transcriptional repressor complex between C/EBPs and HDAC-1 to a specific C/EBP binding site on mouse PPAR/H9252 promoter in keratinocytes.

**DISCUSSION**

The important roles of PPARs and C/EBPs in the regulation of similar pathways in the skin strongly suggest that both families of transcription factors closely interact to regulate keratinocyte proliferation and differentiation. We demonstrate here that C/EBPα and C/EBPβ isotypes, but not C/EBPδ, inhibit the basal activity of the mouse PPARβ promoter in mouse keratinocytes through a mechanism that involves binding to a specific C/EBP response element and recruitment at this site of HDAC-1 that results in histone deacetylation. Most importantly, the expression of PPARβ and C/EBPs are mutually exclusive in keratinocytes from the interfollicular epidermis and hair follicles, suggesting an important role for C/EBP transcription factors in regulating the expression pattern of PPARβ during mouse skin development. Unveiling this interplay leads to a better understanding of the molecular control regulating the balance between differentiation and proliferation in keratinocytes, a balance that is impaired in pathologies such as psoriasis and skin cancers.

**Molecular Mechanism for the C/EBP-mediated Inhibition of PPARβ Expression in Keratinocytes**—Controlled transcriptional repression, like transcriptional activation, has emerged as a mechanism by which tissue-specific gene expression is regulated. Initial characterization of the mouse PPARβ promoter revealed the presence of an AP-1 site that is central for the regulation of PPARβ expression in response to inflammatory cytokines in mouse skin (5, 24). In this study, we present evidence for a novel regulatory mechanism by which C/EBPα and C/EBPβ inhibit basal PPARβ expression in keratinocytes. We have demonstrated that C/EBPα and C/EBPβ bind to a C/EBP response element located in the proximal promoter of the mouse PPARβ gene and recruit a transcriptional repressor complex containing HDAC-1 but lacking HDAC-2. The inhibitory effect on PPARβ expression thus results from deacetylation of histone H4. Binding of C/EBP transcription factors to specific DNA response elements was already reported to be necessary for the inhibition of a number of other genes, including gonadotrophin-releasing hormone (31), liver X receptor-α (27), dentin sialophosphoprotein (32), inhibin α-subunit (33), and trefoil factor-1 (26). However, the underlying molecular mechanisms remained poorly characterized. The model reported here for the inhibition of PPARβ gene expression...
through histone deacetylation might be a general mechanism by which C/EBPs inhibit gene transcription. In favor of this, C/EBPβ was recently reported to inhibit gene transcription via a similar mode of action in preadipocytes (30).

Many additional regulatory mechanisms may influence the effect of C/EBPs on PPARγ expression in mouse skin. However, no phenotypic changes were reported in the skin of C/EBPα-deficient mice, except for a substantial thinning of the subcutaneous fat layer (39). Interestingly, a similar differentiation program was observed in adipocytes, where high levels of C/EBPβ are expressed in the early phase of preadipocyte differentiation, whereas C/EBPα maintains the adipocyte terminal differentiation program (21).

The expression patterns of PPARβ and C/EBPs are mutually exclusive in normal or pathologic conditions in skin. Indeed, PPARβ expression appears in the basal layer of the interfollicular epidermis in mouse skin (4, 6), whereas C/EBPα and C/EBPβ were mainly found in the suprabasal layers. It is note worthy that α2- and α5-integrins, whose expression is restricted to the basal cell layer of the epidermis, are also repressed by C/EBPα and C/EBPβ, C/EBPβ being the more effective repressor in both cases (16). We observed a similar exclusion between PPARβ and C/EBPs in developing postnatal hair follicles, with high PPARβ expression levels in the proliferative follicular epithelial compartment (6), abundant C/EBPα in the dermal compartment, and some C/EBPβ expression in the precortex region. Finally, the expression of C/EBPα was diminished in many cancers, including squamous cell carcinomas and colorectal cancers (12), whereas PPARβ expression levels are frequently increased in similar cancer models (40). Altogether, these data strongly suggest that PPARβ and C/EBPs may have antagonistic functions in keratinocytes and that C/EBPs participate or are responsible for establishing the PPAR expression pattern in mouse skin.
Importance of the Interplay between PPARβ and C/EBPs for the Differentiation of the Epidermis and Hair Follicles—Because less than 1% of C/EBPα-null mice survive after birth, a detailed analysis of the skin phenotype of these mutant mice is difficult to perform (41). In addition, compensatory effects by other C/EBPs may mask hair follicle or other skin abnormalities in the C/EBPα knock-out mice. Indeed, an up-regulation in C/EBPα expression has been reported in keratinocytes of C/EBPβ-deficient mice. However, the absence of C/EBPβ was not totally compensated, since these mice showed a skin phenotype characterized by a scruffy appearance of their coats (13). Detailed analysis of C/EBPα and C/EBPβ expression performed in human and murine adult skin revealed that C/EBPs are expressed in a hair follicle cycling-dependent manner (16). Consistent with our results in the developing hair follicles, high levels of C/EBPα were observed in the fibroblasts of the dermal papilla in murine anagen hair follicle, whereas strong immunoreactivity of C/EBPβ was detected in most outer root sheath cells in the distal hair follicle. The high expression of C/EBPα and C/EBPβ in defined follicular compartments during hair follicle growth strongly suggests that the C/EBP family of transcription factors might be important regulators of hair follicle morphogenesis and cycling.

We previously demonstrated that PPARβ is required for normal postnatal hair follicle development, by regulating the balance between apoptosis and proliferation in follicular keratinocytes (6). The molecular mechanism involves direct activation of the antiapoptotic phosphatidylinositol 3-kinase/Akt1 signaling pathway. Interestingly, the same pathway was also reported to regulate the phosphorylation status of C/EBPs, hence their ability to regulate cell proliferation (12). Antimitotic activities of C/EBPα have been well documented in a variety of cell types and physiological situations (12), including in keratinocytes (15, 19). Growth arrest induced by C/EBPβ is highly cellular context-specific, but several observations indicate an antiproliferative function for this isoform in keratinocytes. It was shown that forced expression of C/EBPβ in BALB/MK keratinocytes inhibits their growth, and mice lacking C/EBPβ exhibit mild epidermal hyperplasia. C/EBPβ-deficient keratinocytes also display resistance to calcium-induced growth arrest, and C/EBPβ expression is diminished in squamous cell carcinomas (14, 15). In addition, C/EBPβ was reported to regulate keratinocyte survival, which is required to complete the differentiation program in the skin (42). Thus, the negative interplay between PPARβ and C/EBPs presented in this study may explain, at least partially, the slight decrease in proliferation observed in PPARβ-deficient follicular keratinocytes of developing hair follicles (6). Another possible explanation for the reduced proliferation of PPARβ-mutant keratinocytes might come from the ability of C/EBP members to modulate the expression of the paracrine factor hepatocyte growth factor in mouse fibroblasts (43). Like C/EBPα, hepatocyte growth factor is specifically expressed in the fibroblasts of the dermal papilla in the developing hair follicle (44), and we already demonstrated that hepatocyte growth factor stimulates PPARβ activity in mouse keratinocytes (6). Thus, it is important to note that, in addition to their ability to regulate the expression level of PPARβ, C/EBPs might also indirectly modulate PPARβ activity in keratinocytes through the regulation of hepatocyte growth factor production within the follicular mesenchyme, allowing for normal hair follicle development.

In summary, this work demonstrates the importance of the regulatory interplay between PPARβ and C/EBP transcription factors in the molecular control of proliferation and differentiation in mouse keratinocytes. Furthermore, the ability of C/EBPα and C/EBPβ to modulate PPARβ expression may have significant impact on the differentiation program in other cell types and subsequently on other processes where C/EBP transcription factors have been involved, including the regulation of liver and lung homeostasis.

Acknowledgments—We thank Véronique Borel, Maria Belen Delgado, Vincent Roh, and Julien Robert for valuable technical help.
Interplay between PPARβ/δ and C/EBPs in Keratinocytes

38. Schuettengruber, B., Simboeck, E., Khier, H., and Seiser, C. (2003) Mol. Cell. Biol. 23, 6993–7004
39. Linhart, H. G., Ishimura-Oka, K., DeMayo, F., Kibe, T., Repka, D., Poindexter, B., Bick, R. J., and Darlington, G. J. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 12532–12537
40. Michalik, L., Desvergne, B., and Wahli, W. (2004) Nat. Rev. Cancer 4, 61–70
41. Flodby, P., Barlow, C., Kylefjord, H., Ahrlund-Richter, L., and Xanthopoulos, K. G. (1996) J. Biol. Chem. 271, 24753–24760
42. Zhu, S., Yoon, K., Sterneck, E., Johnson, P. F., and Smart, R. C. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 207–212
43. Jiang, J. G., and Zarnegar, R. (1997) Mol. Cell. Biol. 17, 5758–5770
44. Lindner, G., Menrad, A., Gherardi, E., Merlino, G., Welker, P., Handjiski, B., Roloff, B., and Paus, R. (2000) FASEB J. 14, 319–332