Suprabasal expression of a dominant-negative RXR α mutant in transgenic mouse epidermis impairs regulation of gene transcription and basal keratinocyte proliferation by RAR-selective retinoids

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To determine whether 9-*cis* retinoic acid receptors (RXRs) regulate the biological activity of all-*trans* retinoic acid (tRA) and its receptors (RARs) in skin, we have targeted a dominant-negative RXR α (dnRXR α) lacking transactivation function AF-2 to differentiated suprabasal keratinocytes in the epidermis of transgenic mice. Driven by the suprabasal-specific keratin-10 gene promoter, expression of dnRXR α severely reduced the ability of RAR-selective ligands tRA and CD367 to induce epidermal mRNA levels of the CRABP II, CRBP I, and CRBP II genes, which contain RA-responsive elements (RAREs) DR1 and/or DR2. It also reduced gene-specific, synergistic induction of CRBP I mRNA by a combination of CD367 and RXR-selective SR11237. Like endogenous RXR α , dnRXR α in epidermal nuclear extracts from the transgenic mice competitively formed heterodimers with endogenous RAR γ on RAREs, suggesting that dnRXR α impairs retinoid signaling by competing with endogenous RAR γ -RXR α heterodimers. Histologically, the epidermis of dnRXR α mice showed no detectable developmental abnormalities. Surprisingly, in adult animals, the suprabasal expression of dnRXR α significantly reduced the ability of topically applied tRA to stimulate proliferation of undifferentiated keratinocytes in the basal layer of epidermis. RXR-selective ligands alone had no detectable effects on both normal and transgenic mouse epidermis. Accordingly, we suggest that *in vivo*: (1) in suprabasal keratinocytes, retinoids regulate gene transcription via RAR-RXR heterodimers in which RAR confers a predominant ligand response, whereas RXR AF-2 is required for liganded RAR AF-2 to efficiently *trans*-activate target genes, and (2) this suprabasal RXR-assisted mechanism indirectly regulates proliferation of basal keratinocytes likely via intercellular signaling.

[Key Words: Retinoids; 9-*cis* retinoic acid receptor; transgenic mouse; epidermal keratinocytes; gene transcription; cell proliferation]

Received September 4, 1996; revised version accepted November 11, 1996.

Vitamin A (retinol) is an important regulator of epithelial cell homeostasis. All-*trans* retinoic acid (tRA) is the major biologically active metabolite of vitamin A. Clinically, tRA and related synthetic retinoids are widely used in the therapy of skin disorders such as cystic acne, psoriasis, photoaging skin, and certain epithelial malignancies (for review, see Peck and Di Giovanna 1994). These effects are thought to be mediated by members of two nuclear receptor gene families, retinoic acid receptor (RAR) genes and 9-*cis* retinoic acid (9cRA) receptor (RXR) genes. Each of the two families comprises three

members: α , β , and γ (for review, see Mangelsdorf and Evans 1995; Chambon 1996; Glass 1996; Pfahl and Chytil 1996). *In vitro* studies have demonstrated that RARs and RXRs form heterodimers (RAR-RXR) or homodimers (RXR-RXR) and stimulate gene transcription by binding to *cis*-acting enhancer elements, termed RA-responsive elements (RAREs), present in gene promoters. The activation function-2 (AF-2) domain at the carboxyl terminus of these receptors confers a ligand-dependent *trans*-activation function by interacting with transcriptional coactivators. RARs recognize both tRA and 9cRA, whereas RXRs interact exclusively with 9cRA. Therefore, RAR-RXR enables cells to respond to both tRA and 9cRA, whereas RXR-RXR enables the response only to 9cRA.

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Previous studies of knockout mice mutated in RAR α and/or RAR γ genes did not reveal any in vivo developmental abnormalities in skin (for review, see Kastner et al. 1995). However, in transgenic mouse models, expression of a dominant-negative (dn) RAR α (dnRAR α) mutant in undifferentiated keratinocytes in the basal layer of epidermis (basal keratinocytes) perturbed epidermal maturation during embryo development and resulted in prenatal lethality (Saitou et al. 1995). Furthermore, expression of a different dnRAR α in differentiated keratinocytes in the suprabasal layers of epidermis (suprabasal keratinocytes) showed reduced epidermal barrier function and high neonatal lethality (Imakado et al. 1995). These models suggest the possible involvement of RARs in epidermal organogenesis and differentiation although the molecular events underlying these biological changes remain unknown.

Epidermal keratinocytes, which represent ~95% of the cell population in epidermis, express RXR α and - β and RAR α and - γ , with RXR α and RAR γ being the predominant species distributed throughout epidermal cell layers (for review, see Fisher and Voorhees 1996). When applied externally to normal adult human and mouse skin, pharmacological doses of tRA induce transcription of retinoid-responsive genes including cellular RA binding protein-II (CRABPII), cellular retinol binding protein (CRBP), and early growth response (egr-1) genes in epidermis. tRA also stimulates proliferation of basal keratinocytes and increases the number of differentiated granular keratinocytes within the suprabasal layers in epidermis, resulting in epidermal thickening. However, it is not known whether these epidermal changes result directly from tRA binding to retinoid receptors within epidermal keratinocytes or indirectly from its action in fibroblasts, which are the major cell population in dermis, another major compartment of skin, and are believed to release paracrine factors to affect epidermis (Marks and Furstenberger 1993). On the other hand, the fact that tRA is partly isomerized to 9cRA in skin (Duell et al. 1996) raises the question as to whether in epidermis in vivo, the topical tRA effects in epidermis are mediated by RAR–RXR and/or RXR–RXR as a result of tRA isomerization. In vitro studies have indicated that in most cases, binding of ligands to the RAR partner is predominant and sufficient for RAR–RXR to *trans*-activate. However, in the absence of RAR ligands, the RXR partner is silent in ligand binding and *trans*-activation once bound to certain RAREs such as DR5 from the human RAR β 2 promoter (Kurokawa et al. 1994; Forman et al. 1995). Therefore, use of RXR-selective retinoids on animal tissues may not allow a full assessment in vivo of whether RXRs participate in retinoid-regulated gene transcription and whether AF-2 of the RXR partner is required for RAR–RXR to *trans*-activate in response to RAR agonists. Also, previous studies of knockout mice mutated in the RXR α gene did not provide any information about the role of RXR α in skin as a result of early embryo lethality (for review, see Kastner et al. 1995).

In this study, we analyzed the role of RXRs in retinoid signaling in skin in vivo using a dnRXR α mutant that

lacks only AF-2. Under the control of a suprabasal keratinocyte-specific promoter from the bovine keratin-10 gene (Bailleul et al. 1990), dnRXR α was targeted to differentiated keratinocytes in the suprabasal layers of transgenic mouse epidermis. In several independent lines of transgenic mice expressing dnRXR α , we found that the stimulatory effects of RAR agonists on transcription of retinoid-responsive genes were markedly reduced in epidermis. Although the dnRXR α mice appeared to have normal epidermal development, the stimulatory effects of the RAR agonists on proliferation of epidermal basal keratinocytes were markedly reduced in the adult transgenic mice. Thus, our study provides for the first time the in vivo evidence that in suprabasal keratinocytes, retinoids regulate gene transcription via RAR–RXR in which RAR confers a predominant ligand response, whereas RXR AF-2 is required for liganded RAR AF-2 to efficiently *trans*-activate. Our data also suggest that the activity of RAR–RXR in suprabasal keratinocytes indirectly stimulates proliferation of basal keratinocytes via intercellular signaling.

Results

Characterization of dnRXR α in receptor dimerization and DNA binding

As RXR α and RAR γ are two predominant retinoid receptor forms in epidermis, we selected an RXR α mutant to interfere with endogenous RXR and/or RAR activity in this tissue. This mutant, termed dnRXR α , contains a carboxy-terminal deletion between amino acid positions 449 and 467 (Fig. 1A). This deletion impairs the AF-2 but not ligand-binding nor dimerization functions of RXR α (Nagpal et al. 1993; Zhang et al. 1994). dnRXR α has been shown to dominantly inhibit ligand-dependent *trans*-activation of RARE-containing reporter genes by wild-type RAR–RXR in cultured cells (Durand et al. 1992; 1994; Xiao et al. 1995). It has also been shown that in vitro transcribed and translated dnRXR α binds to a synthetic thyroid hormone response element (TRE) in the form of RAR β –dnRXR α or dnRXR α –dnRXR α (Zhang et al. 1994). However, the ability of dnRXR α in either heterodimerization with RAR γ on a broad spectrum of naturally occurring RAREs of types DR1, DR2, and DR5 or homodimerization with itself on DR1 has not been documented. To do this, expression vectors for wild-type RXR α or dnRXR α were transfected together with or without those for RAR γ into cultured keratinocytes. Immunological gel mobility shift assays were performed with nuclear extracts prepared from these transfected cells using oligonucleotide probes containing the DR5 from the human RAR β 2 gene, the DR2 from the mouse CRBPI gene, or the DR1 from the rat CRBPII gene.

Because levels of overexpressed receptors far exceed those of endogenous receptors in transfected keratinocytes (Xiao et al. 1995) and because very low amounts (2 μ g) of nuclear extracts from these cells were used, receptor–DNA complexes detected by the assays pertain to

Figure 1. Targeting expression of transgenes to suprabasal keratinocytes in mouse epidermis. (A) Structure of suprabasal keratinocyte-specific expression vectors K10-dnRXR α and K10-lacZ used in preparation of transgenic mice. The arrowhead indicates transcription direction under the control of the bovine keratin 10 (BK-10) gene promoter. The folded line represents intron II from the rabbit β -globin gene. dnRXR α cDNA lacks AF-2, but retains functional domains as indicated. (AF1) Activation function-1; (DNA) DNA-binding domain; (LIGAND) ligand-binding domain; (DIMER) dimerization domain; [SV40 POLY(A)n] a polyadenylation signal sequence from SV40. The size of each DNA fragment is shown below the corresponding structure. Restriction sites [(N) *Not*I; (B) *Bgl*II; (E) *Eco*RI] are also indicated below the constructs. (B) Specific expression of *lacZ* under the control of the BK-10 promoter in epidermal suprabasal keratinocytes. Punch biopsies (4 mm) were taken from the dorsal skin of adult control (CTRL) or K10-lacZ mice, and frozen sections (5 μ m) were prepared. β -Galactosidase activity in keratinocytes (blue) in the suprabasal layers (S) of epidermis was revealed by X-Gal staining of the sections. The sections were briefly counterstained with nuclear fast red. Note that there is no specific blue staining of undifferentiated keratinocytes in the basal layer (B). Bar, 50 μ m.

overexpressed receptors. As shown in Figure 2A, RAR γ -dnRXR α (lane 8) was formed as efficiently as RAR γ -RXR α (lane 2) when DR5 was used as a probe. Mutations in the half-sites of DR5 abolished formation of these dimers (lanes 1 and 7). Like RAR γ -RXR α , RAR γ -dnRXR α was supershifted by monoclonal antibodies against the DE region of RXR α (RXR α -Ab, lane 10) or the amino terminus of RAR γ (RAR γ -Ab, lane 11) and a polyclonal antibody against RARs (RAR-Ab, lane 12). However, monoclonal antibody RXR-Ab, which recognizes a different epitope within the DE region of all three RXR members, did not supershift but rather reduced slightly the formation of RAR γ -dnRXR α (lane 9), in contrast to RAR γ -RXR α that was supershifted by this antibody (lane 3). Similar results were obtained when DR2 or DR1 was used as a probe (Fig. 2B,C). Interestingly, formation

of RAR γ -dnRXR α was more strongly inhibited by RXR-Ab on DR2 (lane 9, Fig. 2B) and DR1 (lane 9, Fig. 2C) than DR5 (lane 9, Fig. 2A).

dnRXR α was also analyzed for its ability in ligand-sensitive homodimerization (Fig. 2D). Like wild-type RXR α (lanes 1 and 2), dnRXR α was able to form homodimers on DR1 (lane 6), and this formation was significantly enhanced by 9cRA (lane 7). Thus, the removal of the 19-amino-acid carboxyl terminus from RXR α does not affect its ligand-sensitive homodimerization function. Interestingly, like DNA-bound RAR γ -dnRXR α , formation of dnRXR α -dnRXR α on DR1 was significantly reduced by RXR-Ab (lane 8), whereas RXR α -Ab supershifted this complex efficiently (lane 9). In comparison, DR1-bound RXR α -RXR α was supershifted by both RXR-Ab (lane 3) and RXR α -Ab (lane 4). These observations indicate that interaction between RXR-Ab and dnRXR α -containing dimers, that is, RAR γ -dnRXR α and dnRXR α -dnRXR α , affects their ability to bind DNA, in marked contrast to RAR γ -RXR α and RXR α -RXR α , respectively. Thus, this antibody can be used to distinguish receptor dimers formed with dnRXR α from those with RXR α .

The above DNA-binding study suggests that dnRXR α is capable of binding to a variety of RAREs as either RAR γ -dnRXR α or dnRXR α -dnRXR α . Thus, if RAR-RXR is the major mediator of retinoid activity on target genes in mouse epidermis, there would be two outcomes from targeting expression of this mutant to the epidermis: (1) If RXR α AF-2 is required for the full function of RAR-RXR, expression of dnRXR α would reduce the ability of retinoids, especially RAR-selective retinoids, to induce transcription of target genes as a result of competitive formation of RAR-dnRXR α on RAREs; and (2) if RXR α AF-2 is dispensable, no changes in the response of epidermis to retinoids would be observed. However, if RXR-RXR mediates retinoid effects in epidermis, RXR-selective retinoids alone would show typical retinoid effects, and expression of dnRXR α would reduce these effects.

Construction of suprabasal keratinocyte-specific expression vectors carrying dnRXR α

To target expression of dnRXR α to the mouse epidermis, we constructed an expression vector K10-dnRXR α (Fig. 1A) using the bovine keratin-10 gene promoter. This full-length promoter has been shown previously to drive expression of many transgenes specifically in differentiated keratinocytes within the suprabasal layers, but not in undifferentiated keratinocytes in the basal layer of mouse epidermis (Bailleul et al. 1990; Werner et al. 1993; Auewarakul et al. 1994). To efficiently produce mRNA of interest, intron II from the rabbit β -globin gene and an RNA polyadenylation signal from the SV40 virus were included in the vector. The parental vector was verified for its ability to direct expression of the bacterial *lacZ* gene in skin. Transgenic mice carrying K10-lacZ showed β -galactosidase activity specifically in suprabasal kera-

Figure 2. Immunological gel mobility shift analysis of the ability of dnRXR α to form either heterodimers with RAR γ on DR5 (A), DR2 (B), and DR1 (C) or homodimers with itself on DR1 (D). Cultured keratinocytes were transfected with a combination of expression vectors for RAR γ , RXR α , or dnRXR α as shown below each gel. Nuclear extracts from these cells (2 μ g) were incubated with 32 P-labeled probes containing wild-type (wt) or mutated (m) DR5, DR2, or DR1 as indicated at the top. Antibodies were added during postincubation as shown immediately above the gels. Types of receptor dimeric complexes identified by antibody supershifting are labeled on both sides of the gels. To stimulate formation of RXR α -RXR α , 1 μ M 9cRA was included in the binding reactions, except for those of lanes 1 and 6 in D.

tinocytes, but not basal keratinocytes, which are located immediately above the dermis (Fig. 1B).

Specific expression of dnRXR α mRNA in the epidermis of transgenic mice

DNA fragments, K10-dnRXR α , were microinjected into fertilized mouse oocytes and transplanted into pseudopregnant female mice (see Materials and Methods). Birth was closely monitored. Newborn mice were screened for the presence of K10-dnRXR α by PCR genotyping using primers specific to the bovine K10 promoter and dnRXR α cDNA. Of these mice, 11% were identified as K10-dnRXR α carriers and did not show developmental abnormalities in skin. We outbred four of these transgenic founders and examined their F1-F4 heterozygous progeny in detail. As shown in Figure 3A, animals from these founder lines carry 5–100 copies of the K10-dnRXR α construct (lanes 2–5), as determined by Southern blotting analysis. To determine whether these mice express dnRXR α , total RNA was extracted from the epidermis of these mice and analyzed by Northern blotting. A 1.9-kb mRNA corresponding to dnRXR α was detected in transgenic mice (Fig. 3B, lanes 2–5), but not in nontransgenic littermates (lane 1). Ratios between dnRXR α and endogenous RXR α vary from 1.5 to 3.5 among these mouse lines. We have also examined tissue distribution of dnRXR α mRNA. As shown in Figure 3C, dnRXR α mRNA is expressed at high levels in epidermis (lane 1)

and to a much lesser extent in tongue (lane 2), but not in other tissues such as liver, heart, lung, kidney, and intestine (lanes 3–7).

Reduced dose-dependent induction of the CRABP1, CRBP1, and CRBP2 genes by all-trans retinoic acid in the epidermis of dnRXR α mice

To know whether expression of dnRXR α led to changes in epidermal responses to retinoids, tRA was applied topically to skin of heterozygous transgenic mice from three independent dnRXR α lines or nontransgenic controls. At 20 hr after treatment, mice were sacrificed and epidermal biopsies were taken immediately. Total mRNA was extracted from the biopsies and analyzed by Northern blotting using CRABP1 cDNA as a probe. We observed 40–70% decreases in induction of CRABP1 mRNA by tRA in transgenic mice as compared with control mice, with mice from line 4539 being the most severely affected, in correlation with relative levels of dnRXR α mRNA expressed in these lines (Fig. 3D). Thus, inhibition of the tRA effect depends on the expression levels but not on the chromosomal integration sites of the K10-dnRXR α transgene. Such inhibition was only slightly enhanced in homozygous 4539 mice (high dnRXR α expressors) because the ratio of dnRXR α to endogenous RXR α was increased from 3.5 in the heterozygotes to only ~4.3 in the homozygotes (data not shown). This limited increase is probably caused by the limited

Figure 3. Characterization of transgenic mice carrying K10-dnRXR α . (A) Southern blotting analysis of genomic DNA (10 μ g) from dnRXR α mice of independent lines (Tg4642, Tg4590, Tg4570, and Tg4539) and a control mouse (CTRL). Signals and sizes corresponding to endogenous RXR α gene or dnRXR α transgene are indicated on the left. The estimated copy number of the dnRXR α transgene in mouse genome is shown immediately below the blot. Genomic DNA from mouse tails was digested with *Bgl*II, which releases the dnRXR α cDNA region (Fig. 1A). (B) Northern blotting analysis of epidermal total RNA (20 μ g) from a control mouse and dnRXR α mice. mRNA signals corresponding to the endogenous RXR α gene or dnRXR α transgene are indicated. mRNA ratios between dnRXR α and endogenous RXR α are shown below the blot. The control signals (β -actin) show variation in the amounts of RNA loaded. (C) Northern blotting analysis of total RNA from different tissues of a dnRXR α mouse. (Top) Autoradiography of a Northern blot containing total RNA (20 μ g) from epidermis (E), tongue (T), liver (L), heart (H), lung (Lu), kidney (K), and intestine (I). (Bottom) The ethidium bromide-stained agarose gel before blotting, which serves as an RNA quality control. Positions of 28S and 18S ribosomal RNA are indicated. mRXR α cDNA was used as a probe in these analyses. (D, top) Autoradiography of Northern blots containing epidermal RNA (20 μ g) from dnRXR α mice of independent lines and a nontransgenic control mouse (CTRL), which were treated topically with vehicle (v) or 80 nmoles tRA (t). (Bottom) A bar graph showing average fold induction of CRABP2 mRNA in tRA-treated mice vs. vehicle-treated mice from the same line. Relative mRNA levels of dnRXR α vs. RXR α in the dnRXR α mouse lines are indicated below the x-axis. (n) Number of mice from each line used in the treatment. Standard errors are indicated on the top of bars.

availability of transcription factors for the high copy number of the bovine K10 promoter in the epidermal cells of transgenic mice.

F1–F4 heterozygous offspring from mouse line 4539 (high dnRXR α expressors) was further analyzed in detail for regulation of CRABP2 in epidermis under acute (20

hr) and chronic (4 days, one application per day) treatments with topical tRA. In the acute treatment, tRA induced CRABP2 mRNA levels much less efficiently in the dnRXR α mice than in the control mice at doses applied (Fig. 4A,B). The inhibitory effect brought by dnRXR α over CRABP2 was also seen in the chronic treatment. Again in correlation with levels of dnRXR α , a moderate reduction in the tRA effect was observed with mice from line 4570 (low dnRXR α expressors) (Fig. 4B). To know whether the inhibitory effects of dnRXR α also extended to other retinoid-responsive genes, epidermal RNA from the tRA-treated mice was analyzed using CRBP-I and -II cDNA as probes. As shown in Figure 4A,C, and D, the effects of tRA on these two genes were reduced significantly in the dnRXR α mice as compared with the control mice. As in the case with CRABP2, the inhibitory effects brought by dnRXR α on these two genes were larger in the chronic treatment than in the acute treatment.

dnRXR α reduced the response of CRABP2, CRBP1, and CRBP2 genes to retinoid receptor-selective agonists

As tRA is isomerized in part to 9cRA in epidermis (Duell et al. 1996), it raises the question of whether tRA exerts its effects on the CRABP2, CRBP1, and CRBP2 genes via the pathways mediated by RAR-RXR and/or RXR-RXR as a result of 9cRA formation. To answer this question, we applied topically the RAR-selective synthetic ligand CD367 at suboptimal doses, RXR-selective synthetic ligand SR11237, or a combination of these two ligands to dnRXR α mouse skin. After 20 hr, epidermal RNA was extracted and analyzed by Northern blotting. As shown in Figure 4E and F, CD367 but not SR11237 alone induced CRABP2, CRBP1, and CRBP2 mRNA significantly in control mice, indicating that induction of these genes by retinoids is mediated by the RAR-, but not the RXR-dependent pathways. Under the same conditions, induction of these genes was ~50–60% lower in the dnRXR α mice, suggesting that RAR-RXR is the mediator of the RAR-dependent pathway and that the integrity of RXR α AF-2 is required for RAR-liganded RAR-RXR to efficiently *trans*-activate. In the control mice, SR11237 synergistically enhanced CD367-induced transcription of CRBP1. This synergism was observed only when CD367 was used at these suboptimal doses. No such synergism was observed with CRABP2 and CRBP2 even when other suboptimal doses of CD367 were used. In the dnRXR α mice, the CD367–SR11237 synergism on CRBP1 was reduced significantly. The residual retinoid induction of these genes may be contributed by the residual activity of *trans*-activation-deficient RAR γ -dnRXR α and/or the activity of remaining RAR γ -RXR α in suprabasal keratinocytes (see Fig. 7, below). Alternatively, as epidermal samples analyzed by Northern blotting contain RNA from both suprabasal and basal keratinocytes, the residual induction might be caused in part by cell types not expressing the transgene, such as basal keratinocytes in the dnRXR α mice.

Figure 4. Reduced tRA effects on transcription of CRABP1, CRBP1, and CRBP2 genes in the epidermis of adult dnRXR α mice. (A) Autoradiography of representative Northern blots containing epidermal RNA from nontransgenic control mice (CTRL, lanes 1–5) and dnRXR α transgenic mice from line 4539 (Tg4539, lanes 6–10), which were treated topically for 1 day (20 hr) with vehicle (VEH, lanes 1 and 6) or tRA at doses indicated above the blots. (B–D) Quantitative comparison of the dose-dependent effects of tRA on induction of CRABP1 (B), CRBP1 (C), and CRBP2 (D) mRNA in control mice with those in dnRXR α mice of lines 4539 or 4570. Mice received either acute (1d, 20 hr) or chronic (4d, 4 days) treatments with topical tRA. The y-axis represents relative mRNA levels in tRA-treated mice expressed as fold induction over the basal level in vehicle-treated mice. Each data point is an average value from a group of mice with a size (*n*) as indicated. The x-axis shows the amount of tRA applied using a log scale. (E) Autoradiography of representative Northern blots containing epidermal RNA from control (CTRL, lanes 1–4) or dnRXR α mice (Tg4539, lanes 5–8), which were treated topically with vehicle (V), 80 nmoles RXR-selective SR11237 (SR), 0.32 nmole RAR-selective CD367 (CD), or SR11237 plus CD367 (CD + SR). Epidermal RNA (20 μ g) from each treated mouse was analyzed by Northern blotting using CRABP1, CRBP1, CRBP2, and β -actin cDNA as probes. (F) Bar graph showing the relative levels of CRABP1, CRBP1, and CRBP2 mRNA in the treated mice. Types of ligands used and genes analyzed are indicated below the x-axis.

dnRXR α reduced the effects of all-trans retinoic acid on keratinocyte proliferation and differentiation in epidermis

tRA induces epidermal hyperplasia characterized by epidermal thickening, hyperproliferation of basal keratinocytes, and expansion of terminally differentiated keratinocytes containing keratohyalin granules, that is, granular keratinocytes. To examine whether dnRXR α affects the ability of tRA to induce epidermal hyperplasia, skin of the dnRXR α mice was treated topically with different doses of tRA for 4 days and examined at histological levels. Nontransgenic littermates were used as controls. As shown in Figure 5, tRA dose-dependently

caused epidermal thickening in the controls. It also increased epidermal cellularity, the volume of cells, space between keratinocytes, and granular keratinocyte layers. Labeling of the epidermis with BrdU revealed a 53% increase in proliferating basal keratinocytes in control mice treated with 8 nmoles tRA for 4 days (Fig. 6). However, in the dnRXR α mice from lines 4539 and 4570, the ability of tRA to induce epidermal thickening was significantly reduced, as compared with the control littermates (Fig. 5A,B). Induction of basal keratinocyte proliferation by 8 nmoles tRA in the dnRXR α mice was significantly lower than that in the controls, although there was no significant difference in proliferation rate among the mice treated with vehicle (Fig. 6). On the other hand,

Figure 5. Reduced epidermal responses to tRA in dnRXR α mice at histological levels. Skin of control (CTRL) or transgenic mice of line 4539 (Tg4539) was treated topically with vehicle (VEH) or tRA at indicated doses for 4 days. (A) Micrographs of sections of mouse dorsal skin showing lower degrees of epidermal thickening, spongiosis, and cellular enlargement in dnRXR α mice (right) as compared with control littermates (left). The sections (5 μ m) were stained with hematoxylin-eosin. Bar, 25 μ m; (E) epidermis; (D) dermis. (B) Reduced dose-dependent induction of epidermal thickness by tRA in dnRXR α mice. (C) Reduced dose-dependent induction of the thickness of granular cell layers by tRA in dnRXR α mice. (B,C) (■) CTRL ($n=4-6$); (▲) Tg4570 ($n=3-4$); (◆) Tg4539 ($n=4-8$).

dnRXR α also reduced the ability of tRA to increase the thickness of granular keratinocyte layers (Fig. 5C). Similar results were obtained with RAR-selective CD367, whereas RXR-selective agonist SR11237 had no effects on both control and dnRXR α mice (data not shown). The tRA-induced epidermal changes were less pronounced in the 4570 mice (low dnRXR α expressors), as compared with the 4539 mice (high dnRXR α expressors) (Figs. 5B,C, and 6B).

Identification of dnRXR α present in keratinocytes of transgenic mouse epidermis as RAR γ -dnRXR α heterodimers

As the epidermis of the dnRXR α mice showed reduced responses to RAR-selective retinoids, it raised the question of whether dnRXR α is able to dimerize with endogenous RARs to form RAR γ -dnRXR α and compete with endogenous RAR γ -RXR α for binding to RAREs in epidermal keratinocytes. We examined keratinocyte nuclear extracts from the epidermis of transgenic heterozygotes and their nontransgenic littermates by immunological gel mobility supershift assays. As shown in Figure 7, formation of complexes with DR5 was observed with the extracts from the control (lane 2) and the trans-

genic mice (lane 8). Mutation of both half-sites in DR5 abolished the complex formation (lanes 1 and 9). These complexes correspond to RAR γ -RXR α in the control mice and comigrating RAR γ -dnRXR α and RAR γ -RXR α in the dnRXR α mice, respectively. RXR-Ab efficiently supershifted endogenous RAR γ -RXR α in both mice, but not RAR γ -dnRXR α in the transgenic mice. In the transgenic mice, increasing amounts of RXR-Ab inhibited binding of RAR γ -dnRXR α to DR5, consistent with the properties of RAR γ -dnRXR α overexpressed in cultured keratinocytes as described above (Fig. 2). Note that a low amount (0.5 μ l) of RXR-Ab was sufficient to supershift RAR γ -RXR α in the dnRXR α mice (lane 10) because increasing amounts of this antibody did not further increase the level of the antibody-associated RAR γ -RXR α complexes (RAR γ -RXR α -RXR-Ab, lanes 11-12). Quantitation of these complexes by PhosphorImager indicates that the ratio between RAR γ -dnRXR α and RAR γ -RXR α in the dnRXR α mice is $\sim 2.5:1$. As expected, both RAR γ -RXR α and RAR γ -dnRXR α were supershifted by RXR α -Ab, which reacts with RXR α and dnRXR α in a similar manner (Fig. 2), and RAR γ -Ab. Similar binding patterns were observed with these extracts when DR1 was used as a probe. No dnRXR α -containing RXR homodimers were detected (data not shown). These data indicate that

Figure 6. Expression of dnRXR α in suprabasal keratinocytes reduces stimulatory effects of tRA on proliferation of basal keratinocytes in transgenic mouse epidermis. (A) Representative micrographs showing BrdU-labeled basal keratinocytes (red) in dorsal skin sections from adult transgenic mice of line 4539 (Tg4539) and nontransgenic control littermates (CTRL) treated with vehicle (VEH) or tRA for 4 days. Bar, 50 μ m; (E) epidermis; (D) dermis. A solid line indicates epidermal–dermal junction. (B) Bar graph showing relative scores of BrdU-labeled basal keratinocytes in the epidermis of transgenic [Tg4539 (solid bar; $n=6-7$) and Tg4570 (hatched bar; $n=4$)] and control mice (open bar; $n=6$). The number of BrdU-labeled basal keratinocytes (KCs) are expressed as percentage of total basal keratinocytes in scored areas. (*) and (**) P value <0.0001 between the transgenic and the control mice, as determined by Fisher's least significant difference test.

at the levels of dnRXR α expressed, both RAR γ -RXR α and RAR γ -dnRXR α were present in keratinocyte nuclei of the transgenic mouse epidermis with RAR γ -dnRXR α being predominant.

Discussion

Previous studies of knockout mice mutated in the RXR α gene have not revealed its role in skin development and homeostasis because of early embryo lethality (for review, see Kastner et al. 1995). Also, analysis of the RXR β knockout mice did not reveal any skin abnormalities probably because of the predominant presence of func-

tionally redundant RXR α in this tissue (Kastner et al. 1996). In this study, our K10-dnRXR α mouse model provides for the first time biological evidence that RXR α is involved in RAR-dependent regulation of gene expression and cell proliferation and differentiation by retinoids in adult epidermis in vivo.

In vitro studies have shown previously that overexpressed RXR–RXR and/or RAR–RXR are able to regulate reporter genes containing RAREs of types DR1 and DR2 from mouse CRABP2, CRBP1, or CRBP2 genes (Smith et al. 1991; Durand et al. 1992; Nakshatri and Chambon 1994). In this study, we found that RAR-selective ligands tRA and CD367, but not RXR-selective ligand SR11237, can induce CRABP2, CRBP1, and CRBP2 mRNA in mouse epidermis in vivo. This finding indicates that the RAR-dependent, but not the RXR-dependent pathways mediate the retinoid regulation of these genes. It also excludes the possibility that the tRA effects are mediated by unidentified RXR–RXR as a result of conversion of tRA to 9cRA in this tissue. Interestingly, we observed a CD367–SR11237 synergism on the CRBP1 gene, which contains a DR2 only. This synergism clearly indicates the presence of the RAR–RXR activity in this tissue. However, no SR11237 effects were observed with CRABP2 and CRBP2 genes, which contain RAREs of both type DR1 and DR2. Thus, the question remained as to whether in epidermis RXRs broadly participate in regulation of gene transcription by RAR-specific retinoids. In vitro studies have shown that binding of ligands to the RAR partner is predominant and is required for RAR–RXR to *trans*-activate RAREs of most DR types. In the absence of RAR ligands, the RXR partner in RAR–RXR bound to certain RAREs, such as DR5 from the hRAR β 2 promoter, is silent in ligand binding and *trans*-activation (Kurokawa et al. 1994; Forman et al. 1995). Therefore, use of RXR-specific agonists alone in animal tissues in vivo would not permit elucidation of the role of RXRs in regulation of certain endogenous genes. Using immunological gel mobility shift assays, we showed that endogenous RAR γ and RXR α , the two predominant retinoid receptors in mouse epidermis, bound to RAREs as heterodimers but not homodimers (Fig. 7) even when DR1 was used as a probe (data not shown). We also showed that expression of dnRXR α lacking AF-2 in suprabasal keratinocytes impaired the induction of CRABP2, CRBP1, and CRBP2 mRNA by RAR-selective ligands. Furthermore, dnRXR α also reduced the CD367–SR11237 synergism on CRBP1.

Together these data strongly suggest that in suprabasal keratinocytes RAR–RXR is responsible for the action of RAR ligands on these genes and that the predominant formation of the heterodimers is likely determined by relative levels of RAR γ versus RXR α . As indicated by our in vitro DNA-binding study, dnRXR α retains its function in dimerization and DNA-binding. Therefore, it most likely dimerizes with endogenous RAR γ in competition with endogenous wild-type RXR α in epidermis. The resulting RAR γ -dnRXR α heterodimers compete with the remaining RAR γ -RXR α heterodimers for binding to RAREs. This interpretation is supported by our

Figure 7. Immunological gel mobility shift analysis of dnRXR α expressed in epidermis of transgenic mice. Epidermal nuclear extracts (6 μ g) from normal control mice (CTRL, lanes 1–7) or dnRXR α mice (Tg4539, lanes 8–14) were incubated with 32 P-labeled probes containing wild-type (wt) or mutated (m) DR5 as indicated at the top. Antibodies RXR-Ab (0.5–3.0 μ l), RXR α -Ab (1.5 μ l), and RAR γ -Ab (1.5 μ l) were added postincubation as shown immediately above the gels. Types of receptor dimeric complexes identified by antibody supershifting are labeled on both sides of the gels.

antibody supershifting experiment with nuclear extracts prepared directly from the dnRXR α mouse epidermis (Fig. 7). Upon binding, RAR γ -dnRXR α most likely does not stimulate transcription of target genes as effectively as RAR γ -RXR α in response to RAR ligands. Consequently, it reduces the retinoid effects on gene transcription. It is noteworthy that DNA-bound RAR γ -RXR α and RAR γ -dnRXR α react differently with monoclonal antibody RXR-Ab. This phenomenon indicates that the conformation of RAR γ -dnRXR α is different from that of RAR γ -RXR α because of the absence of RXR α AF-2. Therefore, reduction by dnRXR α of the RAR-selective ligand effects on gene transcription in vivo may reflect that RXR AF-2 is required for RAR-liganded RAR-RXR to maintain certain conformation necessary for efficiently interacting with transcriptional coactivators. On the other hand, we cannot exclude the possibility that together with liganded RAR AF-2, the AF-2 of unliganded RXR α directly participates in interaction between heterodimers and coactivators.

It has been shown previously that in mouse embryonal carcinoma cell lines F9 and P19, RXR agonists synergize with RAR agonists to induce the endogenous CRABP II gene (Roy et al. 1995). In this study, we observed that in mouse epidermis, RXR-selective ligands do not synergistically enhance induction of CRABP II and CRBP II genes by a suboptimal dose of RAR-selective ligands, although a synergism on the CRBP I gene is present. In the case of the mouse RAR β 2 gene, topical retinoids do not induce its transcription in epidermis (our unpublished observation), in contrast to the F9 and P19 cells (Roy et al. 1995). Thus, regulation of gene transcription by receptor family-selective retinoids and the synergism among these retinoids appear to be both cell type- and gene-specific.

Topical treatment of normal mouse skin with pharmacological doses of tRA increases epidermal thickness, differentiated granular keratinocytes, and proliferating basal keratinocytes in epidermis (Zil 1972; Connor et al.

1986). Gendimenico et al. (1994) have shown previously that topical tRA, but not SR11237 reduced the size of utriculi, a hereditary differentiation defect in the epidermis of rhino mice. We found that in normal mouse epidermis, RAR-selective CD367, but not RXR-selective SR11237 reproduces the tRA-specific effects (J.H. Xiao, X. Feng, Z.H. Peng, W. Di, and J.J. Voorhees, in prep.), consistent with the selective activity of these retinoids on the CRABP II, CRBP I, and CRBP II genes. These observations suggest that an RAR-mediated pathway is responsible for the biological activity of retinoids in epidermis. Here again, the question of whether RXRs are involved in this pathway remained unanswered. In this study, we showed that expression of dnRXR α in suprabasal keratinocytes significantly reduced the retinoid effects on epidermal proliferation and differentiation. Thus, this RAR-dependent pathway most likely involves RXRs in the form of RAR-RXR heterodimers. In particular, the integrity of the major *trans*-activating domain AF-2 of unliganded RXR α appears to be necessary for the heterodimers to fully mediate the epidermal responses to tRA. Significant histological changes in mouse epidermis can be observed initially on day 2 during the chronic retinoid treatment, whereas the transcriptional event exemplified by induction of CRABP II, CRBP I, and CRBP II mRNA occurs within 24 hr after the initial topical application and persists throughout the chronic treatment. Together, these observations suggest that a transcriptional process is likely required prior to cellular changes. In the dnRXR α mice, the residual tRA effects on epidermal proliferation and differentiation are likely caused by the residual activity of RAR γ -dnRXR α and the activity of remaining endogenous RAR γ -RXR α , in agreement with the residual induction of the retinoid-responsive genes. Although to date, no data have suggested a direct role of CRABP II, CRBP I, and CRBP II in regulation of epidermal proliferation and differentiation, the molecular mechanism governing their retinoid responsiveness

may also apply to as yet unidentified retinoid target genes that mediate the molecular events leading to epidermal changes.

Disruption of *RAR α* and/or *RAR γ* genes in mice did not affect normal skin development although overall growth deficiency, high postnatal lethality, and malformation of some other organs were observed with these mice (Kastner et al. 1995). Similar to these *RAR* knockout mice, our *dnRXR α* mice did not display any significant abnormalities in epidermal structure or cellularity during development. Interestingly, a significant increase of resistance to *tRA*-induced malformations at day 8.5 or 9 postcoitus was observed with the *RAR γ* knockout mice (Kastner et al. 1995). At the adult stage, skin of these mice exhibits a remarkable resistance to the systemic effects of excessive *tRA* injected intraperitoneally such as skin scaling and hair loss (Look et al. 1995). Su-cov et al. (1995) have reported that at the embryo stage, limbs of the *RXR α* knockout mice developed normally, but showed resistance to the teratogenic effects of the *tRA* treatment. These phenotypes are somewhat similar to the reduced proliferative response of epidermis to topical *tRA* in the adult *dnRXR α* mice. Thus, *RAR*–*RXR* is likely responsible for these cellular changes induced by exogenous retinoids. In the *dnRXR α* mice, the residual activity of *RAR γ* –*dnRXR α* and the activity of the remaining endogenous *RAR γ* –*RXR α* appear to be sufficient to support epidermal development and homeostasis under endogenous retinoid status.

In epidermis, keratinocytes in the basal layer undergo proliferation to give daughter cells that lose proliferative potential and subsequently undergo differentiation while ascending to suprabasal layers (Fig. 8). Growth factors and cytokines released from different epidermal cell types and other skin compartments such as dermis are thought to affect proliferation of basal keratinocytes. To assess these possibilities, expression vectors made of differentiation-specific gene promoters such as the *K10* gene have been developed to target expression of growth-related proteins to differentiated suprabasal keratinocytes. For example, expression of the HPV16 E6 and E7 oncogenes in the suprabasal keratinocytes of mouse epidermis led to overexpression of *TGF α* in these cells and concomitant hyperproliferation of basal keratinocytes (Auewarakul et al. 1994). Werner et al. (1993) have shown that suprabasal expression of a dominant-negative FGF receptor mutant caused an increase in proliferation of basal keratinocytes. Retinoids applied topically to mouse skin were shown to rapidly reach epidermal and dermal cells (Connor et al. 1985). However, it has not been made clear whether retinoids stimulate proliferation of basal keratinocytes directly via receptors within these cells or indirectly via those in suprabasal keratinocytes or dermal fibroblasts. On the other hand, the retinoid signaling pathway in basal cells may possibly coordinate with that in other cell types to produce the hyperproliferative response. Here, we report that expression of *dnRXR α* in suprabasal keratinocytes reduced the proliferative response of basal keratinocytes to topically applied *RAR*-selective retinoids. Thus, the biological

Figure 8. A proposed model for regulation of basal keratinocyte proliferation by retinoids. Structure of the epidermis is schematically depicted. Epidermal keratinocytes undergo differentiation from stem cells in the basal layer to mature corneocytes, resulting in stratum corneum. Suprabasal keratinocytes consist of spinous and granular cells, which are in the early and late stages of differentiation, respectively. The suprabasal cells express filament proteins keratin-1 and -10 (K-1 10) instead of keratin-5 and -14 (K-5 14) specific to the basal cells. Topically applied retinoids activate target genes via *RAR*–*RXR* heterodimers present in the nucleus of suprabasal cells which in turn send signals to induce proliferation of the basal cells.

cal activity of *RAR*–*RXR* heterodimers in the suprabasal cells appears to regulate proliferation of basal cells. This finding suggests for the first time that via the *RAR*–*RXR*-mediated pathway, retinoids induce biological changes in suprabasal keratinocytes, which in turn stimulate basal keratinocytes to proliferate by releasing as yet unidentified signaling factors and/or cell–cell contacts (Fig. 8). Further characterization of the *K10*–*dnRXR α* mouse model will be necessary to identify molecules involved in this process. Also, expression of *dnRXR α* within basal keratinocytes will be required to ascertain whether the activity of *RAR*–*RXR* heterodimers within basal cells is required for responding to the suprabasal signal.

Materials and methods

Overexpression of RAR γ , RXR α , and dnRXR α in cultured keratinocytes

RAR γ , *RXR α* , and *dnRXR α* were overexpressed by transfection of cultured human epidermal keratinocytes with expression vectors pSG5–*mRAR γ* , pSG5–*mRXR α* , or pSG5–*mRXR α dn*, and nuclear extracts were prepared from these cells as described previously (Xiao et al. 1995).

Immunological gel mobility shift assays

Oligonucleotide probes containing wild-type or mutated DR5 from h*RAR β 2* and wild-type DR1 from r*CRBP*II were described previously (Xiao et al. 1995). Sequences for probes containing wild type or mutated DR2 from the m*CRBP*I promoter (Smith et al. 1991) are: 5′-tcgacTTTAGTAGGTCAAAAGGTCAGACAC-3′ (consensus hexameric half-sites shown in boldface capi-

tal letters and randomly chosen nucleotides in lowercase) and 5'-tcgacTTTAGTTTGTCAAAAGGTTGGACAC-3' (mutations underlined), respectively. Double-stranded oligonucleotide probes were 5'-end-labeled using [γ - 32 P]ATP, and immunological gel mobility shift assays were performed as described previously (Xiao et al. 1995). Antibodies used in this study were mouse monoclonal antibodies RXR-Ab (4RX1D12), RXR α -Ab (4RX3A2) (Rochette-Egly et al. 1994), and RAR γ -Ab [Ab1 γ (A1)] (Rochette-Egly et al. 1991), and a rabbit polyclonal antibody RAR-Ab [RAR γ (M454)] (Santa Cruz Biotechnology). Amounts equivalent to undiluted antibody fluids used in most cases were 1–2 μ l.

Construction of epidermis-specific expression vectors for dnRXR α and lacZ

To construct K10-dnRXR α , a polycloning site 5'-NotI-BgIII-SalI-AvaI-StuI-EcoRI-HindIII-BamHI-XhoI-NotI-3' was inserted into pBlueScript SK(-) (Stratagene) between SstI and KpnI sites to give pMCS-1. A double-stranded oligonucleotide spacer was prepared by annealing oligonucleotides, 5'-TCGGAATTGTGAGCGCTCACAATTC-3' and 5'-CCGAGAATTGTGAGCGCTCACAATT-3', and inserted into the AvaI site in pMCS-1 to give pLBS. Then, a 850-bp StuI-SalI fragment containing rabbit β -globin gene intron-II, a polycloning site (5'-EcoRI-BamHI-BgIII-3'), and the SV40 polyadenylation signal sequence, was isolated from pSG5 and inserted into pLBS between the StuI and XhoI sites, resulting in pLBS-2. To obtain the bovine keratin-10 (BK10) gene promoter, pCKVI-CAT was digested with SalI and XhoI (Bailleul et al. 1990). A 4.8-kb fragment containing the BK10 promoter, which extends from a SalI site to a KpnI site 20 bp downstream of the TATA box, was purified and inserted into the SalI site in pLBS-2 to give pXJ81L. Finally, a 1.5-kb EcoRI-BgIII fragment from pSG5-mRXR α dn (Durand et al. 1992), which contains dnRXR α cDNA, was inserted into pXJ81L between the EcoRI and BamHI sites, resulting in pXJ81L-mRXR α dn.

To construct K10-lacZ, a 3.9-kb HindIII-SalI fragment containing the bacterial lacZ gene coding sequence and the SV40 polyadenylation signal was liberated from pXJ40-lacZ (Xiao et al. 1991), and inserted into pLBS between HindIII and XhoI sites to give pLZ. A 0.7-kb StuI-HindIII fragment containing the rabbit β -globin gene intron II was isolated from p513, which is identical to pSG5 except for its polycloning site (5'-EcoRI-BamHI-HindIII-XhoI-NotI-SmaI-PstI-SstI-KpnI-BgIII). This fragment was inserted into pLZ between the StuI and HindIII sites, resulting in pLGZ. Finally, the BK10 promoter fragment was inserted into the SalI site to give pXJ81L-lacZ.

Preparation of transgenic mice

K10-dnRXR α (7.7 kb) and K10-lacZ (9.4 kb) were liberated from plasmid vectors pXJ81L-mRXR α dn and pXJ81L-lacZ, respectively, by digestion with NotI, separated by electrophoresis on a 0.8% agarose gel, and purified through a Nucleobond AX20 column (The Nest Group). The purified DNA fragment was microinjected into F2 hybrid zygotes from C57BL/6J $\times$ SJL/J parents (the Jackson Laboratory) at a concentration 2–3 ng/ μ l (Hogan et al. 1994). After overnight incubation, the eggs which survived to the two-cell stage were transferred to day 0.5 postcoitum pseudopregnant CD-1 females (Charles River). Founder transgenic mice were mated to C57BL/6J mice to give F1 offspring. F2–4 offspring was produced by crossing F1–F3 transgenic mice with C57BL/6J mice. The microinjection was performed by the Transgenic Animal Model Core of the University of Michigan's Biomedical Research Core Facilities. All procedures using mice

were approved by the University of Michigan Committee on Use and Care of Animals, and were conducted in accord with the principles outlined in the NIH guidelines for the care and use of experimental animals.

Identification of transgenic mice by PCR genotyping

Transgenic mice carrying K10-dnRXR α or K10-lacZ were identified by PCR genotyping of tail DNA using primers specific to the BK10 promoter, dnRXR α cDNA, or lacZ. Mouse tail DNA was prepared as described previously (Hogan et al. 1994). PCR amplification was carried out for 30 cycles using 200–400 ng of tail DNA, with each cycle being at 94°C for 1.5 min; 60°C for 2 min; and 72°C for 3 min.

Southern blotting analysis of genomic DNA from transgenic mice

Genomic DNA from mouse tails was digested with appropriate restriction enzymes (Sambrook et al. 1989). Digestion products were separated by agarose gel electrophoresis and transferred to Zeta-Probe GT blotting membranes (Bio-Rad). cDNA probe preparation, prehybridization, hybridization, and washing were carried out as described for Northern blotting (see below).

Topical retinoid treatment

Retinoids including tRA, CD367, and SR11237 were obtained as described previously (Xiao et al. 1995). Adult mice (6–8 weeks old) were fed with laboratory rodent diet #5001 (PMI Feeds Inc.). Prior to retinoid treatment, skin hairs were removed using a Golden A-5 clipper (Oster Professional Products). A single daily dose (800 μ l) of retinoids dissolved in 100% acetone (vehicle) was applied topically to the entire mouse skin for a period of 1 (20 hr) or 4 days, which are referred to as acute or chronic treatments, respectively.

cDNA probes, preparation of mouse epidermal RNA, and Northern blotting analysis

Mouse CRABPII cDNA was provided by Dr. V. Giguere (Giguere et al. 1990). To prepare CRBPI (421 bp, positions 1–421, Smith et al. 1991), CRBPII (420 bp, positions 49–468) (Nakshatri and Chambon 1994), and β -actin (245 bp, positions 105–349) (Stratagene) cDNA, mouse epidermal RNA was reverse-transcribed and amplified using a RT-PCR kit (Stratagene) and appropriate primers. The resulting DNA fragments were subcloned into the PCRII vector (Invitrogen) and verified by sequencing. cDNA probes were labeled with [α - 32 P]-dCTP (3000 Ci/mmol) (DuPont-NEN) using a random priming kit (Life Technologies).

To prepare epidermal RNA, mice were sacrificed by cervical dislocation. Skin biopsies were taken using a keratome device with blade depth set as 0.2 mm to cut near the epidermis-dermis junction. Epidermal biopsies were immediately snap-frozen in liquid nitrogen. Then, total RNA was prepared from epidermis using guanidine isothiocyanate followed by a CsCl gradient (Elder et al. 1991). Northern blotting analysis of purified epidermal total RNA was performed as described previously (Xiao et al. 1991). Autoradiography was carried out by exposing blots to Hyperfilm MP (Amersham Life Science) at -70°C overnight. For quantitation of mRNA, the blots were analyzed with a PhosphorImager. mRNA signals were quantified using software ImageQuant (Molecular Dynamics), and normalized against corresponding retinoid-insensitive β -actin signals. Then, the signal values from retinoid-treated mice were converted to fold of induction over vehicle-treated mice in the same

experiment. A different mouse in the group represents a sample number (n) of one. Northern blotting data presented in this study pertain predominantly to RNA from epidermal cells as a smaller form of β -actin mRNA specifically expressed in dermal cells was not detected in epidermal RNA preparation.

Histological examination of mouse epidermis and in situ immunostaining of BrdU-labeled keratinocytes in mouse epidermis

Punch biopsies (4 mm) were taken from the dorsal skin of mice and fixed in a 10% neutral buffered formalin solution (Biochemical Sciences). The specimens were then embedded in paraffin, sectioned at 5 μ m, and stained with hematoxylin-eosin by American Histolabs. Thickness of epidermal and granular keratinocyte layers was measured with an ocular micrometer at fifteen interfollicular areas for each mouse.

To detect proliferating cells in epidermis, mice were injected intraperitoneally with 5-bromo-2'-deoxyuridine (BrdU, 50 mg per kg of body weight) (Sigma) after the topical retinoid treatment. After 1 hr, the mice were killed immediately by cervical dislocation, and 4 mm punch biopsies were taken from dorsal skin and embedded in an OCT compound, Tissue-Tek (Miles Laboratories). Frozen sections (5 μ m) were prepared from the specimens using a cryostat, and stained with peroxidase-conjugated monoclonal antibodies against BrdU (Boehringer Mannheim). Peroxidase activity was revealed using 3-amino-9-ethylcarbazole (Sigma). The sections were then briefly counterstained with Gill's hematoxylin (Biochemical Sciences). To determine the number of BrdU-labeled basal keratinocytes, four punch biopsies (4 mm) were taken from the dorsal skin of each treated mouse. Three frozen sections were prepared from each biopsy. The numbers of BrdU-labeled basal cells and total basal cells in each section were counted with 2–4 high power fields (0.0625 mm²) in interfollicular areas. For each mouse, the numbers from all four biopsies were pooled, and the percentage of the BrdU-labeled basal cells over the total basal cells (>800) was calculated.

Acknowledgments

We thank Drs. M. Blessing for kindly providing us the bovine keratin 10 promoter, V. Giguere for mCRABP II cDNA, B. Shroot for CD367, and B. Janssen for SR11237. We are grateful to Dr. T. Saunders for preparation of transgenic mice, Dr. G. J. Fisher for helpful discussions, A. A. Bowen, N.-Z. Zhou, and L. Qin for technical assistance, L. Van Goor for illustrations, and T. Hamilton for statistical analysis. This work was supported by the Babcock Dermatological Research Fund and a grant from the Johnson & Johnson Corporation. J.-H.X. is a recipient of Career Development Award from the Dermatology Foundation.

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X Feng, Z H Peng, W Di, et al.

Genes Dev. 1997, **11**:

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