

# Identification of heparin-binding EGF-like growth factor as a target in intercellular regulation of epidermal basal cell growth by suprabasal retinoic acid receptors

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**The role of retinoic acid receptors (RARs) in intercellular regulation of cell growth was assessed by targeting a dominant-negative RAR $\alpha$  mutant (dnRAR $\alpha$ ) to differentiated suprabasal cells of mouse epidermis. dnRAR $\alpha$  lacks transcriptional activation but not DNA-binding and receptor dimerization functions. Analysis of transgenic mice revealed that dnRAR $\alpha$  dose-dependently impaired induction of basal cell proliferation and epidermal hyperplasia by all-trans RA (tRA). dnRAR $\alpha$  formed heterodimers with endogenous retinoid X receptor- $\alpha$  (RXR $\alpha$ ) over RA response elements in competition with remaining endogenous RAR $\gamma$ -RXR $\alpha$  heterodimers, and dose-dependently impaired retinoid-dependent gene transcription. To identify genes regulated by retinoid receptors and involved in cell growth control, we analyzed the retinoid effects on expression of the epidermal growth factor (EGF) receptor, EGF, transforming growth factor- $\alpha$ , heparin-binding EGF-like growth factor (HB-EGF) and amphiregulin genes. In normal epidermis, tRA rapidly and selectively induced expression of HB-EGF but not the others. This induction occurred exclusively in suprabasal cells. In transgenic epidermis, dnRAR $\alpha$  dose-dependently inhibited tRA induction of suprabasal HB-EGF and subsequent basal cell hyperproliferation. Together, our observations suggest that retinoid receptor heterodimers located in differentiated suprabasal cells mediate retinoid induction of HB-EGF, which in turn stimulates basal cell growth via intercellular signaling. These events may underlie retinoid action in epidermal regeneration during wound healing.**

**Keywords:** cell proliferation/epidermis/HB-EGF/retinoid X receptor/transgenic mouse

## Introduction

Vitamin A and its metabolites (retinoids), such as all-trans retinoic acid (tRA), are important regulators of embryo development, cellular activity and tissue homeostasis. Their biological effects are mediated by two families of nuclear hormone receptors, retinoic acid (RAR) and retinoid X (RXR) receptors, which are ligand-dependent transcription factors (Kastner *et al.*, 1995; Chambon, 1996; Minucci and Ozato, 1996; Pfahl and Chytil, 1996; Nagy *et al.*, 1997; Perlmann and Evans, 1997). Each receptor family consists of three members,  $\alpha$ ,  $\beta$  and  $\gamma$ . The two receptor families display distinct ligand specificities. Natural retinoids, tRA and its isomer 9-cis RA, are both recognized by RARs, whereas RXRs interact with 9cRA but not with tRA. In the form of RAR-RXR or RXR-RXR dimers, the receptors act through binding to retinoic acid response elements (RAREs) present in the transcriptional regulatory region (promoter) of target genes. Upon retinoid binding, the dimers stimulate gene transcription through interaction with transcriptional co-activators and thereby confer positive retinoid responsiveness. In the absence of retinoids or presence of inverse agonists, the receptor dimers interact with co-repressors and repress basal transcription of target genes (Chambon, 1996; Klein *et al.*, 1996; Shibata *et al.*, 1997; Torchia *et al.*, 1998).

The effects of retinoids on cell growth have been observed in cells cultured *in vitro*. Surveys of >200 cell lines have indicated that retinoids either stimulate or inhibit cell proliferation depending on cell types (Amos and Lotan, 1990; Rogers, 1997). Notably, retinoids inhibit proliferation of many fast-growing transformed cell lines, but promote growth of most primary cells. Studies of mutant cell lines clearly indicated that RAR and RXR members are required for retinoids to inhibit proliferation and promote differentiation of transformed embryonic cells *in vitro* (Chiba *et al.*, 1997, and references therein). Although genes directly associated with cell cycle progression were found to be up- or down-regulated by retinoids in cultured cells, the molecular cascades connecting retinoid receptors to these downstream genes have yet to be established (Rogers, 1997). On the other hand, in epithelial tissues *in vivo*, retinoids such as tRA were also found to either increase or reduce cell proliferation, depending upon the physiological state of host tissues (Peck and Di Giovanna, 1994; Fisher and Voorhees, 1996; Rogers, 1997). For example, treating normal adult human and mouse skin with pharmacological doses of vitamin A or tRA increases the proliferation rate of undifferentiated cells that constitute the basal layer of the epidermis (basal keratinocytes), leading to an accelerated turnover of epidermal cells and thickening of the epidermis. However, in abnormal epithelial tissues such as psoriatic skin, retinoids mitigate hyperproliferation of abnormal

epidermal cells. RAR $\gamma$ , RAR $\alpha$ , RXR $\alpha$  and RXR $\beta$  were detected in skin, with RAR $\gamma$  and RXR $\alpha$  being the predominant species (Fisher and Voorhees, 1996). Although the molecular events underlying retinoid actions in skin have not been elucidated, retinoid receptor-mediated signaling within dermal fibroblasts and/or epidermal basal keratinocytes was suspected to be involved in regulation of epidermal growth (Peck and Di Giovanna, 1994). Unexpectedly, a previous study of mouse skin expressing a dominant-negative RXR $\alpha$  mutant indicated that retinoid regulation of basal cell proliferation involves RXRs in differentiated suprabasal cells of the epidermis (suprabasal keratinocytes) (Feng *et al.*, 1997). Yet, the roles of RARs and retinoid-targeted genes involved in regulation of basal cell proliferation have not been elucidated.

Here, we have examined the roles of RARs in retinoid regulation of cell growth by targeting a dominant-negative RAR $\alpha$  mutant (dnRAR $\alpha$ ) to the differentiated suprabasal layers of epidermis. Analysis of transgenic mouse epidermis revealed that dnRAR $\alpha$  dose-dependently suppressed tRA induction of the basal cell proliferation and epidermal thickening. As a result of formation of transactivation-inactive dnRAR $\alpha$ -RXR $\alpha$  heterodimers, dnRAR $\alpha$  dose-dependently impaired the endogenous RAR-RXR heterodimer-mediated epidermal retinoid signaling pathway at the level of gene transcription. Most interestingly, we found that tRA caused an early and selective induction of one epidermal growth factor (EGF) family member, heparin-binding EGF-like growth factor (HB-EGF), which is a paracrine/juxtacrine growth factor and is expressed exclusively in the differentiated suprabasal layers of the epidermis. This suprabasal-specific HB-EGF induction was inhibited *in situ* by dnRAR $\alpha$ . Our data strongly suggest that RAR-RXR located in suprabasal cells mediates retinoid induction of HB-EGF, which in turn stimulates basal cell growth via paracrine/juxtacrine mechanisms.

## Results

### **Establishment of stable transgenic mouse lines expressing the dnRAR $\alpha$ transgene**

Mouse cDNA encoding dnRAR $\alpha$  was inserted into a cell type-specific transgene expression vector (Figure 1A), which has been constructed previously based on the bovine keratin-10 gene promoter (Feng *et al.*, 1997). This promoter has been used to target expression of a variety of transgenes specifically to differentiated keratinocytes constituting the suprabasal layers of the epidermis (Feng *et al.*, 1997, and references therein). dnRAR $\alpha$  contains a C-terminal deletion between amino acids 404 and 462, which removes ligand-dependent transcription activation function-2 (AF-2) and a 9cRA-binding site but not the tRA-binding, receptor dimerization and DNA-binding functions (Damm *et al.*, 1993; Durand *et al.*, 1994). DNA fragments containing the K10-dnRAR $\alpha$  transgene were isolated and microinjected into fertilized mouse eggs. Out of 71 pups born, no stillborn pups were observed, and 13 (18%) were identified as transgenic mice by PCR genotyping of their tail DNA (data not shown). At birth, six of 13 transgenic founders displayed shiny skin. One severely affected founder had sticky skin and was sacrificed due to the lack of care from its foster CD-1 mouse (Figure 1B). The shiny

skin phenotype is similar to that observed with RAR $\gamma$  null mice (P.Kastner and P.Chambon, unpublished observation) and transgenic FVB mice in which a human dnRAR $\alpha$  was targeted to epidermis using the human keratin-1 gene promoter (Imakado *et al.*, 1995). However, similarly to the RAR $\gamma$  null mice, the shiny skin phenotype dissipated within 1 week after birth. At adult stages, all transgenic founders had normal epidermal cellularity and structure.

Six transgenic founders were chosen and back-crossed with wild-type C57BL/6 mice to establish stable transgenic lines. The F<sub>1</sub> transgenic progeny were analyzed by Southern blotting analysis of tail DNA. As shown in Figure 1C, the copy number of the K10-dnRAR $\alpha$  transgene in these six lines varies from three to 58. At birth, the skin of F<sub>1</sub> transgenic pups from lines 4522, 4518 and 4489, but not 4512, 4487 and 4511, was shiny to different extents, like their founders, with lines 4518 and 4489 being the most prominent, in correlation with levels of dnRAR $\alpha$  expressed (Figure 1D). Northern analysis of total epidermal mRNA from four of these lines showed that expression ratios between dnRAR $\alpha$  and endogenous wild-type RAR $\alpha$  were between 1.7 and 14 (Figure 1D). As expected, expression of dnRAR $\alpha$  mRNA was restricted to the epidermis (Figure 1E). Like their founders, the shiny skin phenotype of the F<sub>1</sub> transgenic pups dissipated within 1 week after birth and, at adult stages, their epidermal cellularity and structure appeared to be normal.

### **Suprabasal expression of dnRAR $\alpha$ inhibited induction of epidermal hyperplasia and basal cell proliferation by tRA in transgenic mouse skin**

To investigate the effects of dnRAR $\alpha$  expression on epidermal growth responses to retinoids, we expanded mouse colonies from three transgenic lines (4522, 4518 and 4489) that express dnRAR $\alpha$  at different levels. Back skin of adult control or transgenic mice was treated with vehicle or tRA for 4 days, and skin sections were examined by hematoxylin-eosin staining. As shown in Figure 2A, tRA significantly increased epidermal cellularity and thickness in control mice. The tRA effects were drastically inhibited in transgenic mice although epidermal structure and cellularity appeared to be similar between these mice without the tRA treatment. The degree of inhibition (Figure 2B) correlated well with the levels of dnRAR $\alpha$  expressed in these three lines (Figure 1D), with complete inhibition in line 4489 that expresses the highest level of dnRAR $\alpha$  (Figure 2A). To ascertain whether dnRAR $\alpha$  affected the ability of tRA to induce basal keratinocyte proliferation, we injected control and transgenic mice intraperitoneally with 5-bromo-2'-deoxyuridine (BrdU). Proliferating cells that have incorporated BrdU into their genomic DNA were visualized by staining skin sections with antibodies specific to BrdU. As shown in Figure 2C, tRA significantly increased the number of proliferating basal keratinocytes in control but not transgenic epidermis. Again, the reduction of tRA-induced cell proliferation (Figure 2D) was in good correlation with levels of dnRAR $\alpha$  expressed in these transgenic lines (Figure 1D). These observations suggest that disruption of endogenous retinoid signaling by dnRAR $\alpha$  in differentiated suprabasal keratinocytes impaired an intercellular signaling pathway(s) through which retinoids regulate basal cell proliferation.

**dnRAR $\alpha$  in transgenic mouse epidermis impaired endogenous RAR $\gamma$ -RXR $\alpha$  heterodimer-mediated gene transactivation through formation of heterodimers with endogenous RXR $\alpha$**

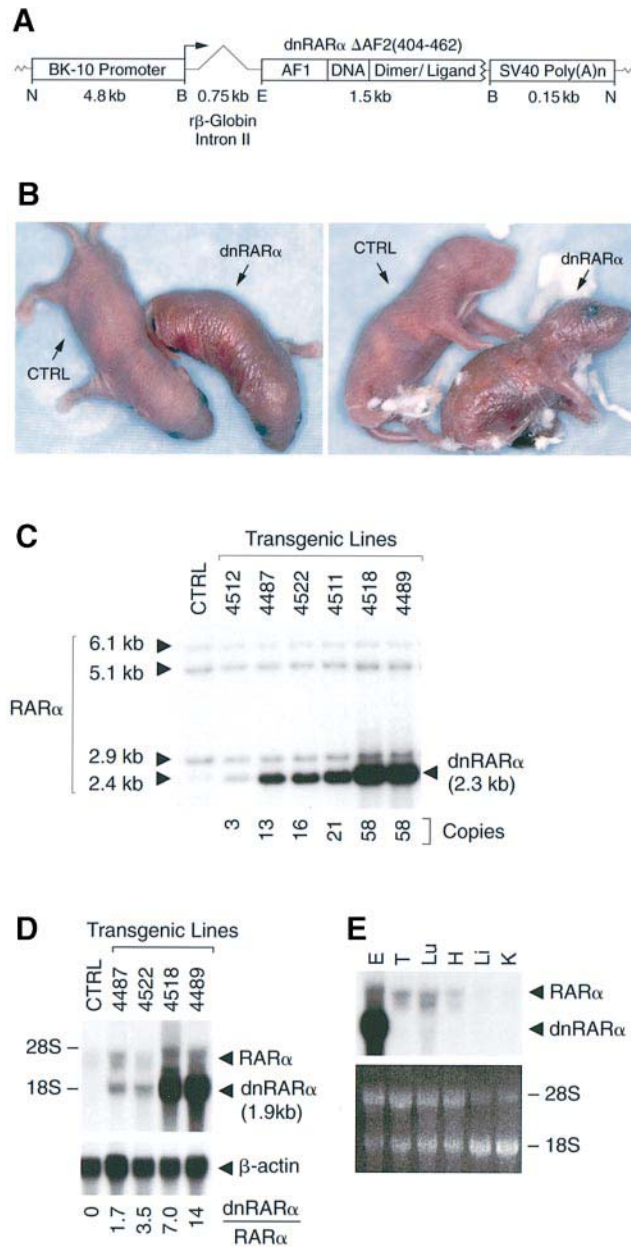
*In vitro* studies have indicated that dnRAR $\alpha$  is capable of repressing gene transactivation by wild-type RAR-RXR heterodimers through competitive formation of DNA-binding-proficient and transactivation-deficient heterodimers, dnRAR $\alpha$ -RXR $\alpha$  (Damm *et al.*, 1993; Durand *et al.*, 1994). By gel mobility supershift analysis, we examined whether dnRAR $\alpha$  in transgenic epidermis was able to heterodimerize competitively with endogenous RXR $\alpha$ , the predominant member of the RXR family in skin (Fisher and Voorhees, 1996). As shown in Figure 3, in normal mouse epidermis, endogenous RAR $\gamma$  and RXR $\alpha$  bound to RARE as heterodimers, which were supershifted by antibodies specific to RAR $\gamma$ , RXR or RXR $\alpha$ , but not RAR $\alpha$ . RAR $\alpha$ -RXR $\alpha$  heterodimers were not detected by gel supershift analysis (Figure 3), probably due to low levels of RAR $\alpha$  expression in normal epidermis

(Figure 1D; see also Fisher and Voorhees, 1996; Feng *et al.*, 1997). In transgenic mouse epidermis, dnRAR $\alpha$ -RXR $\alpha$  heterodimers were detected by antibodies specific to RAR $\alpha$  or RXR $\alpha$ , in addition to remaining RAR $\gamma$ -RXR $\alpha$  heterodimers. These observations indicate that like dnRXR $\alpha$  (Feng *et al.*, 1997), dnRAR $\alpha$  expressed in transgenic epidermis is capable of interfering with the function of RAR $\gamma$ -RXR $\alpha$  heterodimers, the predominant retinoid receptor form in epidermis, through formation of dnRAR $\alpha$ -RXR $\alpha$  heterodimers.

To ascertain whether formation of dnRAR $\alpha$ -RXR $\alpha$  functionally disrupted endogenous RAR-RXR heterodimer-mediated retinoid signaling at the level of gene transactivation in transgenic epidermis, we analyzed tRA effects on expression of the cellular retinoic acid-binding protein II gene (CRABP II), whose promoter harbors two RAREs (Durand *et al.*, 1992). As shown in Figure 4, both basal and tRA-induced levels of CRABP II mRNA were significantly reduced in transgenic epidermis. The degree of inhibition of the tRA effects (Figure 4) correlated well with relative levels of dnRAR $\alpha$  in the transgenic lines (Figure 1D). Thus, dnRAR $\alpha$  effectively interfered with endogenous RAR-RXR activity in gene transactivation in transgenic epidermis.

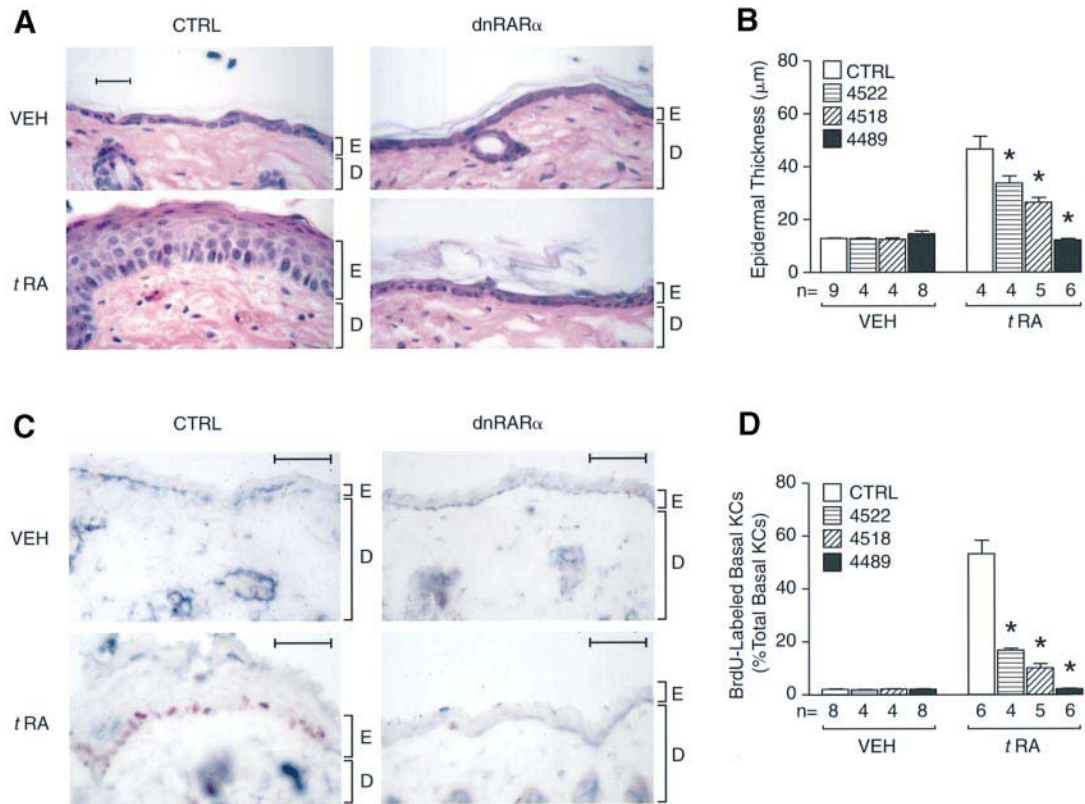
**Early and selective induction of one member of the EGF family, HB-EGF, by tRA in normal mouse epidermis**

Epidermal growth is regulated by ligands binding to epidermal growth factor receptor (EGFR) present on basal keratinocyte membranes (King *et al.*, 1990; Vassar and Fuchs, 1991). The EGFR ligands including EGF, transforming growth factor- $\alpha$  (TGF- $\alpha$ ), HB-EGF and amphiregulin are known to stimulate keratinocyte proliferation



**Fig. 1.** Characterization of the K10-dnRAR $\alpha$  transgenic mice.

(A) Structure of the K10-dnRAR $\alpha$  transgene. The arrowhead shows the transcriptional direction in the bovine keratin 10 (BK-10) gene promoter. The folded line represents the intron II of the rabbit  $\beta$ -globin gene. dnRAR $\alpha$  cDNA lacks AF-2, but retains functional domains as indicated. AF1, activation function-1; DNA, DNA-binding domain; Ligand, tRA-binding domain; Dimer, dimerization domain. SV40 Poly(A)n, a polyadenylation signal sequence derived from SV40. The size of each DNA fragment is shown below the corresponding segment. Restriction sites, *NotI* (N), *BglII* (B) and *EcoRI* (E), are labeled immediately below the constructs. (B) Photograph of newborn normal and transgenic littermates. Left: a picture of transgenic founder 4489 (dnRAR $\alpha$ ) with a normal non-transgenic littermate (CTRL). Right: a picture of the most severely affected transgenic founder with a normal non-transgenic littermate. (C) Southern blotting analysis of tail genomic DNA from the F<sub>1</sub> mice from transgenic lines, 4512, 4487, 4522, 4511, 4518 and 4489, and a non-transgenic control mouse (CTRL). DNA (5  $\mu$ g) was digested by *BglII*. DNA fragments corresponding to the endogenous RAR $\alpha$  gene or the dnRAR $\alpha$  transgene are indicated on both sides of the blot. The estimated dnRAR $\alpha$  transgene numbers in the mouse genome are shown immediately below the blot. (D) Northern blot analysis of epidermal total RNA (20  $\mu$ g) from a control mouse and F<sub>1</sub> mice from transgenic lines, 4487, 4522, 4518 and 4489. mRNA signals corresponding to the endogenous RAR $\alpha$  gene or dnRAR $\alpha$  transgene are indicated. mRNA ratios between dnRAR $\alpha$  and endogenous RAR $\alpha$  are indicated immediately below the blot. The control signals ( $\beta$ -actin) show integrity and variation in the amounts of RNA samples loaded. (E) Northern blotting analysis of total RNA from different tissues of a dnRAR $\alpha$  mouse (line 4489). Upper panel: autoradiography of a Northern blot containing total RNA (20  $\mu$ g) from epidermis (E), tongue (T), lung (Lu), heart (H), liver (L) and kidney (K). Lower panel: photograph of the ethidium bromide-stained agarose gel before blotting, which serves as an RNA quality control.



**Fig. 2.** dnRAR $\alpha$  expressed in transgenic epidermis dose-dependently impaired induction of epidermal thickness and basal cell proliferation by tRA. Skin of adult mice was treated with vehicle (VEH, acetone) or 8 nmol of tRA (in acetone) for 4 days with one topical application per day, and punch biopsies (2 mm) of dorsal skin were taken for histological analysis. n refers to the number of mice analyzed. E, epidermis; D, dermis. (A) Representative micrographs of skin sections showing lack of epidermal expansion, spongiosis and cellular volume enlargement in dnRAR $\alpha$  mice (line 4489) treated with tRA, in comparison with normal control littermates (CTRL) under the same treatment. Dorsal skin sections (5  $\mu$ m) were stained with hematoxylin–eosin. Bar, 25  $\mu$ m. (B) Quantitation of the dnRAR $\alpha$  effect on tRA induction of epidermal thickening in transgenic mice from independent lines, 4522, 4518 and 4489 that express different levels of dnRAR $\alpha$  (4522<4518<4489). The y-axis shows epidermal thickness. Data are mean  $\pm$  SE. \* $p$  < 0.05 versus tRA-treated control mice, as determined by Dunnett's *t* test. (C) Representative micrographs of skin sections labeled with BrdU, which show lack of proliferating basal keratinocytes in the dnRAR $\alpha$  mice (line 4489) treated with tRA. The sections were stained with peroxidase-conjugated antibodies against BrdU. Cells with red staining are proliferating basal keratinocytes, which were revealed by the antibody staining. Bar, 50  $\mu$ m. (D) Quantitation of levels of BrdU-incorporated basal keratinocytes in transgenic mice from independent lines 4522, 4518 and 4489 that express different levels of dnRAR $\alpha$  (4522<4518<4489) and control non-transgenic littermates (CTRL). The y-axis represents relative levels of BrdU-labeled basal cells expressed as a percentage of total basal cells. Data are mean  $\pm$  SE. \* $p$  < 0.05 versus tRA-treated control mice.

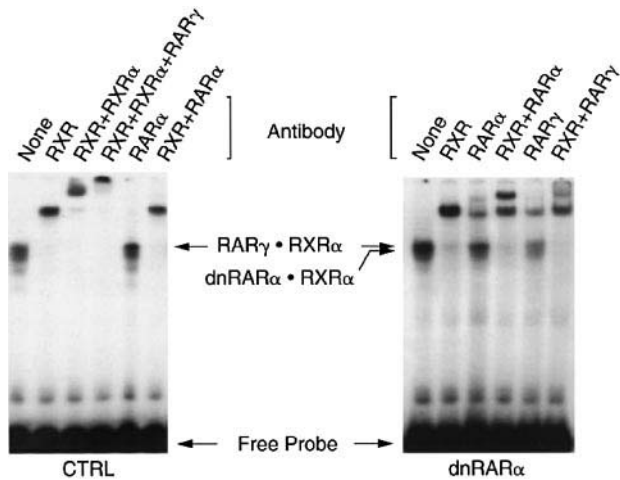
(Raab and Klagsbrun, 1997; Stoll *et al.*, 1997). In normal skin, keratinocytes express very low levels of these factors. Overexpressing or treating skin with these factors causes epidermal hyperproliferation (Vassar and Fuchs, 1991; Dominey *et al.*, 1993; Stoll *et al.*, 1997). To determine whether genes encoding EGFR and its ligands are regulated by retinoids, we analyzed their mRNA levels in normal mouse epidermis treated with vehicle or tRA. Figure 5A shows that tRA did not significantly alter expression of EGFR mRNA and protein. EGF mRNA was not detectable by Northern blotting analysis although it was detected by RT-PCR analysis. However, tRA did not increase the levels of EGF mRNA (data not shown). No significant induction of TGF $\alpha$  by tRA was observed (Figure 5B and C). Interestingly, HB-EGF mRNA was induced rapidly by tRA, reaching a maximal level ~6 h after tRA application (Figure 5B and C). Its induction profile is similar to that of CRABP II. We also found that amphiregulin mRNA was not significantly altered by tRA in normal mouse epidermis (Figure 5C). These data indicate that tRA is able to induce selectively one member of the EGF family, HB-EGF.

**dnRAR $\alpha$  significantly impaired tRA induction of HB-EGF in differentiated suprabasal cells of transgenic epidermis**

To determine whether reduction in epidermal responses to tRA in transgenic mice is associated with dnRAR $\alpha$  inhibitory activity over HB-EGF, we compared HB-EGF mRNA levels between normal and transgenic epidermis. Figure 6 shows that the ability of tRA to induce HB-EGF mRNA was significantly impaired in the transgenic mice. The degree of this impairment correlated well with the relative levels of dnRAR $\alpha$  expressed in these transgenic mouse lines. A similar impairment in retinoid induction of HB-EGF mRNA was observed with K10-dnRXR $\alpha$  transgenic mice (data not shown).

As dnRAR $\alpha$  was targeted to suprabasal keratinocytes, it raised the question as to whether the affected retinoid regulation of HB-EGF occurs directly within suprabasal cells or indirectly in basal cells. We analyzed expression of HB-EGF mRNA in normal and transgenic skin by *in situ* hybridization. At 8 h after tRA application, significant induction of HB-EGF was observed exclusively in epidermal suprabasal keratinocytes of normal skin



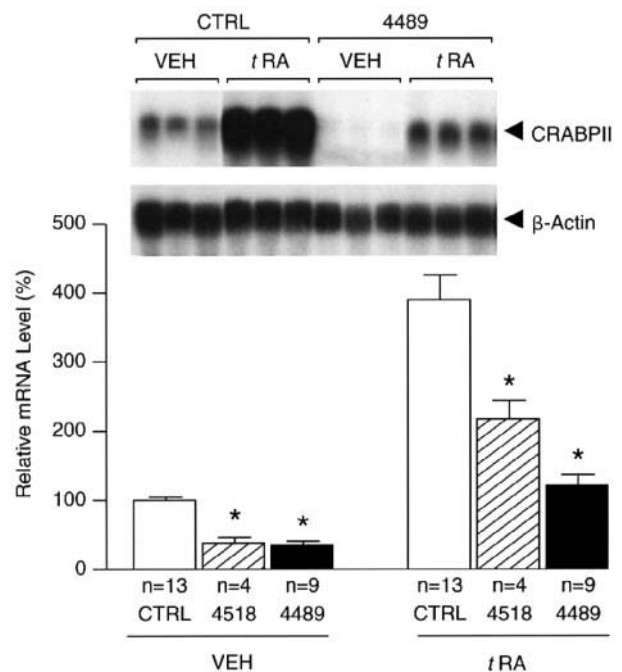


**Fig. 3.** dnRAR $\alpha$  in nuclear extracts of transgenic mouse epidermis competitively formed heterodimers with endogenous RXR $\alpha$  over RARE. Gel mobility supershift analysis was performed with 6  $\mu$ g of nuclear extracts from epidermis of normal control mice (CTRL) or transgenic mice (dnRAR $\alpha$ , line 4489).  $^{32}$ P-labeled oligodeoxyribonucleotide duplexes containing a RARE (DR5) from the human RAR $\beta$ 2 gene promoter (de The *et al.*, 1990) were used as probes. Antibodies (1.5  $\mu$ l) against all three members of the RXR family (RXR-Ab), RXR $\alpha$ , RAR $\gamma$  or RAR $\alpha$ , were added during post-incubation as shown immediately above the gels. The types of receptor dimeric complexes identified by antibody supershifting are labeled along the gels.

(Figure 7A). Epidermal basal cells and dermal fibroblasts did not show detectable levels of HB-EGF mRNA. In transgenic skin, tRA induction of HB-EGF mRNA expression in suprabasal cells was severely impaired. Sustained induction of HB-EGF mRNA was observed in differentiated suprabasal keratinocytes of normal but not transgenic epidermis treated with tRA for 4 days (Figure 7B). Note that epidermal hyperplasia was observed only in control mice treated with tRA for 4 days. Again, no specific HB-EGF mRNA signal was observed in basal keratinocytes and dermal fibroblasts

## Discussion

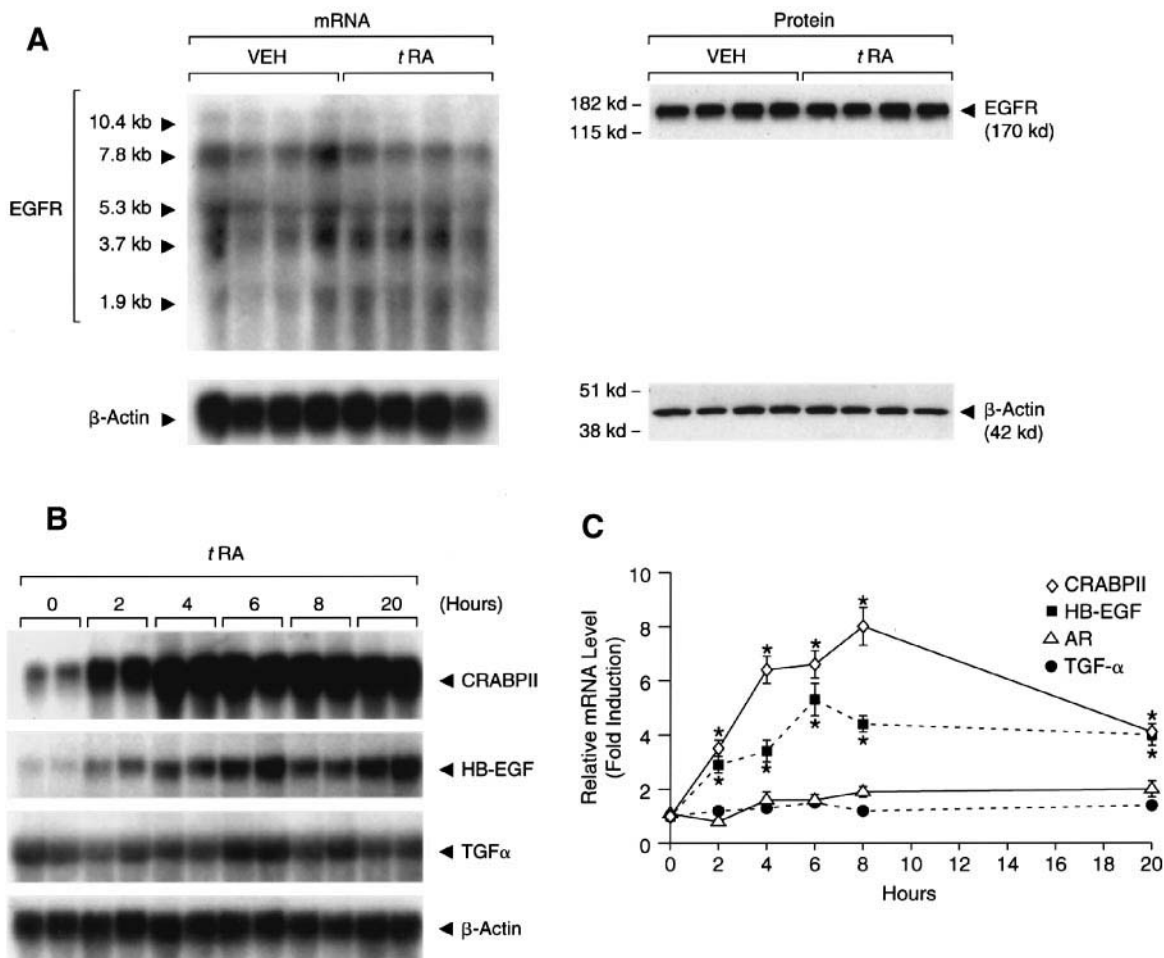
Involvement of RAR–RXR or RXR–RXR dimers in the anti-proliferative effects of retinoids has been suggested by studies of cell lines mutated in the receptor genes or overexpressing wild-type or mutated receptors (Frangioni *et al.*, 1994; Clifford *et al.*, 1996; Chiba *et al.*, 1997; Isogai *et al.*, 1997; Wan *et al.*, 1998). On the other hand, studies of mouse models, in which genes encoding retinoid receptors or their coactivators are inactivated, have indicated their importance in normal cell growth in organogenesis during embryo development (Kastner *et al.*, 1995; Yao *et al.*, 1998). However, little is known about the molecular cascades linking retinoid receptors to cell growth and especially how retinoids stimulate cell growth. Results from this study suggest for the first time that *in vivo* regulation of basal cell growth by retinoids in epidermis involves retinoid receptor-mediated induction of a paracrine/juxtacrine growth factor gene, *HB-EGF*, within differentiated suprabasal cells.



**Fig. 4.** dnRAR $\alpha$  dose-dependently inhibited tRA induction of the CRABP II mRNA levels in transgenic mouse epidermis. Skin of adult mice was treated with vehicle (VEH, acetone) or 40 nmol of tRA (in acetone) for 20 h. Epidermal mRNA was analyzed by Northern blot using mouse CRABP II cDNA as a probe. Top: Northern blot containing epidermal total mRNA (20  $\mu$ g) from normal control (CTRL) or transgenic mice (line 4489). Bottom: quantitation of CRABP II mRNA levels in the normal control and transgenic mice from lines 4518 and 4489 that express different dnRAR $\alpha$  levels (4518>4489). The CRABP II mRNA levels are expressed as a percentage of its basal levels in the vehicle-treated normal control mice, after being normalized against the control  $\beta$ -actin signal in the same sample. Data are mean  $\pm$  SE. \* $p$  < 0.05 versus control mice under the same treatment.

### Role of suprabasal RARs in mediating retinoid induction of basal cell growth in epidermis

In skin, keratinocytes constitute ~95% of the epidermal cell population. Basal keratinocytes are located immediately above the basement membrane that separates the epidermal and dermal compartments. These undifferentiated cells possess the potential to proliferate to give daughter cells, which lose contact with the basement membrane and the ability to proliferate while moving up to suprabasal layers and undergoing differentiation. In adult mouse skin, ~2% of basal cells are proliferating at any given time (Figure 2D; Feng *et al.*, 1997). Retinoids applied externally to skin increase the number of basal cells entering into the proliferative phase. In epidermis of K10–dnRAR $\alpha$  mice, the stimulatory effect of tRA on basal cell growth was impaired dose-dependently by dnRAR $\alpha$  targeted to suprabasal cells (Figure 2). This phenomenon indicates that the activity of endogenous suprabasal RARs is required for basal cells to respond to retinoids. Disruption of such activity by dnRAR $\alpha$  impairs the ability of retinoids to induce growth signaling from suprabasal cells to basal cells, resulting in a lack of epidermal hyperplasia in K10–dnRAR $\alpha$  mice. Saitou *et al.* (1995) have targeted a dnRAR $\alpha$  to basal cells using the keratin-14 gene promoter. They observed that disruption of retinoid signaling by dnRAR $\alpha$  in basal cells inhibited fetal skin development. The skin of affected K14–dnRAR $\alpha$  animals had neither



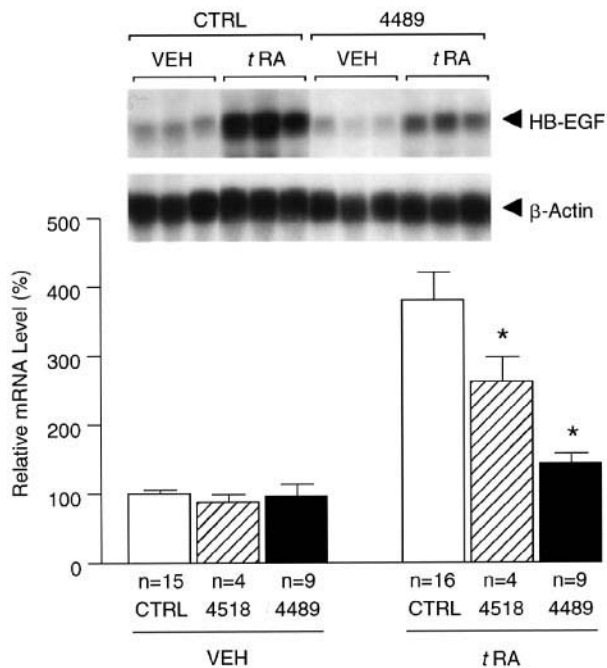
**Fig. 5.** tRA rapidly and selectively induced mRNA levels of one member of the EGF family, HB-EGF, in normal mouse epidermis. Skin of adult mice was treated with vehicle (VEH, acetone) or 40 nmol of tRA (in acetone). (A) Epidermis of normal mice was treated with for 20 h and subjected to the following analyses. Left: Northern blot analysis of EGFR mRNA. Right: Western blot analysis of EGFR protein. The positions of pre-stained molecular weight markers for proteins (Bio-Rad Laboratories, Hercules, CA) are indicated to the left of the Western blot. The same blots were probed for  $\beta$ -actin, which was used as a control for the integrity of mRNA and proteins and loading variation. Each lane represents one individual mouse that received the treatment indicated. (B) Northern blot analysis of CRABP II, HB-EGF and TGF- $\alpha$  expression in normal mouse epidermis treated with tRA. Epidermal biopsies were taken at the indicated times (h) after the tRA application. (C) The time course of tRA induction of CRABP II, HB-EGF, TGF- $\alpha$  and amphiregulin (AR) mRNA in epidermis of normal mice. The y-axis represents relative mRNA levels expressed as fold induction over basal levels in vehicle-treated mice. The x-axis shows time of sampling after tRA application. Data are mean  $\pm$  SE ( $n > 5$ ) and were normalized against the  $\beta$ -actin mRNA signal. \* $p < 0.05$  versus the vehicle-treated mice.

wrinkles nor hairs, was very thin (<30–70% of normal skin thickness) and dried rapidly. The animals died within a few hours after birth. These phenotypes were not observed in the K10–dnRAR $\alpha$  mice, suggesting that basal cell RARs play an important role during skin development whereas suprabasal RARs mediate the epidermal hyperproliferative response to retinoids in adult skin. Together with the fact that suprabasal expression of dnRXR $\alpha$  blocked the hyperproliferative response of basal cells to retinoids (Feng *et al.*, 1997), our observations strongly support the idea that suprabasal RAR–RXR is the functional unit mediating retinoid signaling in intercellular regulation of basal cell growth.

**Impairment of RAR $\gamma$ –RXR $\alpha$  heterodimer-mediated gene transactivation by dnRAR $\alpha$  in transgenic mouse epidermis**

*In vitro* analysis has shown that dnRAR $\alpha$  lacking its AF-2 is capable of binding RAREs as heterodimers with RXRs (Damm *et al.*, 1993; Durand *et al.*, 1994). However, such

dimers do not transactivate in the presence of retinoids, due to their inability to interact with co-activators. In addition, their interaction with retinoids fails to release co-repressors, in contrast to wild-type dimers (Chen and Evans, 1995). Thus, dnRAR $\alpha$  inhibits retinoid-dependent gene transactivation mediated by wild-type RAR–RXR through competing with wild-type RARs for RXR partners and binding to RAREs. Consistent with its properties *in vitro*, this study shows that dnRAR $\alpha$  in transgenic epidermis formed dnRAR $\alpha$ –RXR $\alpha$  heterodimers in competition with RAR $\gamma$  (Figure 3). The functional impact of dnRAR $\alpha$  on gene transactivation was demonstrated by its ability to inhibit transactivation of the CRABP II gene by endogenous retinoid receptors (Figure 4). Gel supershifts showed that RAR $\gamma$ –RXR $\alpha$  is the predominant receptor form interacting with RAREs in normal epidermis (Figure 3), consistent with previous findings (Xiao *et al.*, 1995; Feng *et al.*, 1997). Although low levels of endogenous RAR $\alpha$  mRNA were present in the epidermis (Figure 1D), endogenous RAR $\alpha$ –RXR $\alpha$  heterodimers



**Fig. 6.** dnRAR $\alpha$  dose-dependently inhibited tRA induction of the HB-EGF mRNA levels in transgenic epidermis. Northern blot analysis of HB-EGF expression was performed with the same samples in Figure 4 using HB-EGF cDNA as a probe. The HB-EGF mRNA levels are expressed as a percentage of its basal levels in the vehicle-treated normal control mice, after being normalized against the  $\beta$ -actin signal. Data are mean  $\pm$  SE. \* $p$  < 0.05 versus tRA-treated control mice.

were not detected (Figure 3). Therefore, RAR $\gamma$ -RXR $\alpha$  most likely dominates regulation of RARE-containing genes such as CRABP II, consistent with previous findings (Feng *et al.*, 1997). However, we cannot exclude the possibility that the minute amount of endogenous RAR $\alpha$ -RXR $\alpha$  contributes in part to tRA effects in epidermis.

In K10-dnRAR $\alpha$  mice, the basal level of CRABP II transcription in epidermis was found to be lower than that in the normal control mice (Figure 4). This reduction is probably caused by the mutation-increased ability of dnRAR $\alpha$ -RXR $\alpha$  to interact with nuclear receptor co-repressors (Chen and Evans, 1995). Such a reduction, however, was not observed in K10-dnRXR $\alpha$  mice (Feng *et al.*, 1997). One possible explanation for this difference between the two types of mice is that in untreated dnRXR $\alpha$  mouse epidermis, co-repressors were bound less tightly to RAR $\gamma$ -dnRXR $\alpha$  than to dnRAR $\alpha$ -RXR $\alpha$  in untreated dnRAR $\alpha$  mouse epidermis. Unlike the CRABP II gene, basal transcription of the HB-EGF gene was not significantly affected by dnRAR $\alpha$ . This finding raises the possibility that repression of basal transcription by dnRAR $\alpha$ -RXR $\alpha$  may be gene specific, depending on whether retinoid receptors are involved directly or indirectly in regulation of HB-EGF transcription (see Discussion below).

#### Cell-specific expression and regulation of HB-EGF by RAR-RXR heterodimers *in vivo*

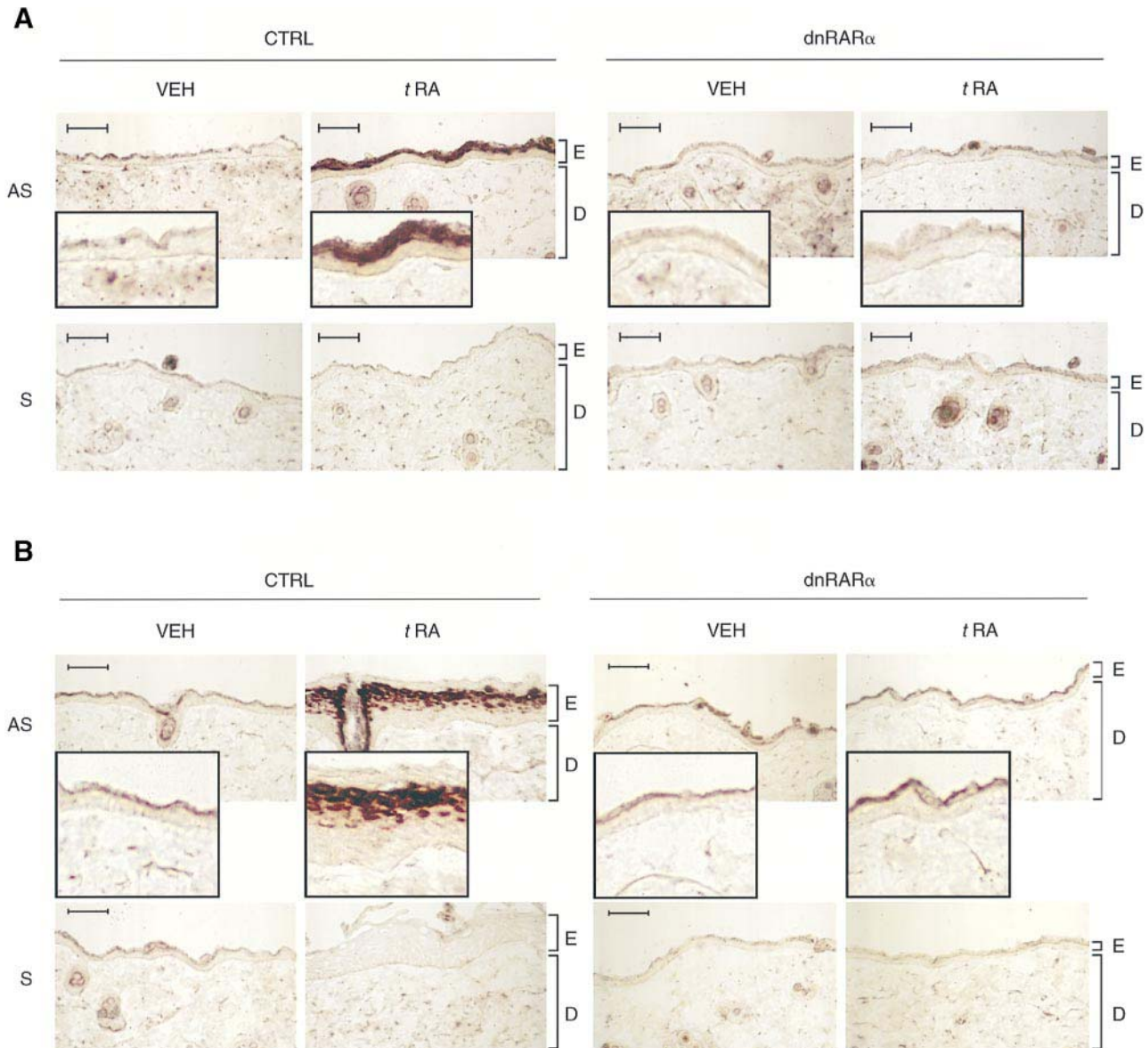
The expression pattern of HB-EGF transcripts in skin *in vivo* (Figure 7) is similar to that of the endogenous K-10 gene (data not shown), specific to differentiated suprabasal keratinocytes. No expression of HB-EGF in

dermal fibroblasts and basal keratinocytes was detected by *in situ* hybridization (Figure 7), indicating that its gene is silent in these cells. Its transcription is most likely regulated by suprabasal-specific transcription factors. Keratinocytes cultured *in vitro* are considered to be similar to basal keratinocytes based on their ability to proliferate and express basal cell-specific genes such as keratin-5 and -14. In these cultured cells, basal expression and autocrine regulation of HB-EGF have been observed (Hashimoto *et al.*, 1994). This difference between *in vitro* and *in vivo* observations suggests that HB-EGF is subject to different regulation mechanisms in basal keratinocytes in skin *in vivo* versus keratinocytes cultured *in vitro*.

As shown by Northern analysis, tRA induced HB-EGF but not other members of the EGF family in normal epidermis (Figure 5). The time course profile of the induction mirrors that of the RARE-containing gene, CRABP II (Figure 5). In transgenic mice, this induction was impaired by dnRAR $\alpha$  or dnRXR $\alpha$  targeted to the suprabasal cells (Figures 6 and 7; and data not shown). Together, these observations strongly suggest that RARs in the form of RAR-RXR heterodimers play a key role in regulation of the HB-EGF gene. Examination of the published sequence of the HB-EGF gene promoter (Chen *et al.*, 1995) failed to reveal any putative RAREs. It is possible that this gene contains as yet unidentified RAREs upstream or downstream of the previously reported promoter region. Alternatively, the HB-EGF gene may be regulated by a retinoid-responsive transcription factor(s) that binds to its transcriptional regulatory regions. Further analysis of the HB-EGF gene sequence and characterization of its transcriptional regulatory regions will be required to distinguish among these possibilities.

#### Role of retinoid receptor-regulated HB-EGF in intercellular regulation of basal cell growth by retinoids

Unlike *in vitro* cell culture systems, in adult animal tissues *in vivo*, stem or undifferentiated cells undergo cell division at a low rate to give daughter cells, and simultaneously regenerate themselves. These cells co-exist and communicate constantly with differentiating daughter cells. This study revealed for the first time that retinoids are able selectively to regulate one member of the EGF family, HB-EGF, within the differentiated cell compartment (Figure 7). HB-EGF is a heparin-binding member of the EGF superfamily. In many cell types including keratinocytes, HB-EGF signals through EGFR to induce cell proliferation and motility by paracrine, juxtacrine or autocrine mechanisms (Raab and Klagsbrun, 1997). In normal skin, mature HB-EGF proteins have been localized to epidermal basal cells (Downing *et al.*, 1997). However, our study revealed that low basal levels of HB-EGF mRNA are present only in suprabasal but not basal keratinocytes nor dermal fibroblasts in normal skin (Figure 7). This location difference suggests that HB-EGF is produced by suprabasal cells and translocates to interact with EGFR on the basal cell surface (King *et al.*, 1990; Vassar and Fuchs, 1991) to maintain normal cell growth. The HB-EGF induction occurs within differentiated suprabasal keratinocytes (Figure 7A) prior to the onset of basal keratinocyte hyperproliferation (Figures 2 and 7B). The dose-dependent inhibitory effects of dnRAR $\alpha$  on tRA induction of



**Fig. 7.** Expression of dnRAR $\alpha$  in transgenic mouse epidermis impaired retinoid induction of HB-EGF mRNA by tRA exclusively within differentiated suprabasal cells. *In situ* hybridization analysis of HB-EGF expression was performed with skin sections from normal control (CTRL) and transgenic mice (dnRAR $\alpha$ , line 4489) using digoxigenin-labeled HB-EGF riboprobes. Alkaline phosphatase-conjugated antibodies specific to digoxigenin were used to reveal hybridization signals. HB-EGF mRNA is stained dark purple. Dorsal skin was treated with vehicle (acetone, VEH) or 20 nmol of tRA for 8 h (A) or 4 days (B) before the analysis. Bar, 50  $\mu$ m. AS refers to skin sections (upper micrographs) subjected to hybridization using the antisense probe. S refers to skin sections (lower micrographs) analyzed with the sense probe, which serves as a control for background signals.

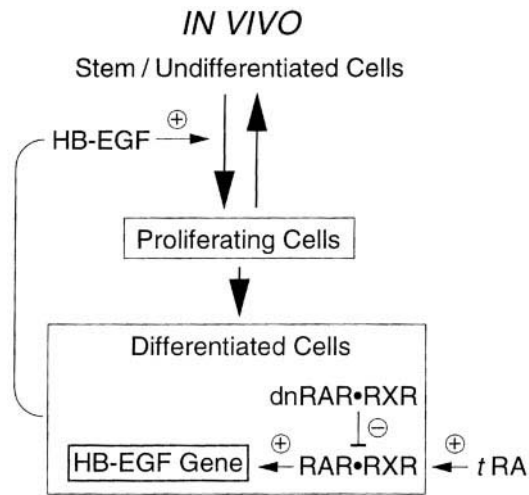
HB-EGF correlated well with its inhibitory effects on the tRA induction of basal cell proliferation. Our data suggest that retinoids induce HB-EGF expression via RAR–RXR in differentiated suprabasal cells, and the resultant high levels of HB-EGF cause basal keratinocyte hyperproliferation, most probably through paracrine or juxtacrine mechanisms (Figure 8), and consequently epidermal hyperplasia. Induction of HB-EGF by tRA *in vivo* is unlikely to trigger its autoregulation observed in cultured keratinocytes (Hashimoto *et al.*, 1994) because its gene is silent in basal keratinocytes in epidermis and because differentiated suprabasal cells, in which the HB-EGF gene is active, do not have EGFR to respond to its autocrine signaling. Consistent with this idea, no induction of HB-EGF in basal keratinocytes nor suprabasal cell proliferation

was observed in skin treated with tRA for 4 days (Figures 2C and 7B).

**Potential roles of retinoid receptor-regulated HB-EGF in the retinoid effects on epidermal regeneration during wound healing**

A previous study has shown that in skin wound sites where basal cell proliferation is increased, levels of HB-EGF proteins were found to be elevated in differentiated suprabasal keratinocytes (McCarthy *et al.*, 1996). On the other hand, it has been known for over half a century that vitamin A deficiency causes delayed wound healing (Brandaleone and Papper, 1941; Hunt, 1970). Pretreatment with vitamin A and tRA was reported to improve skin wound healing in animals and humans (Strigini and





**Fig. 8.** A proposed *in vivo* model for regulation of cell proliferation in the undifferentiated cell compartment by retinoids acting on the differentiated cell compartment in epidermis.

Ryan, 1996 and references therein). As retinoid induction of HB-EGF occurs prior to the onset of basal cell hyperproliferation, and both can be inhibited by dnRAR $\alpha$  (Figure 7), it raises the possibility that HB-EGF may be implicated in retinoid actions in epidermal regeneration during wound healing. In this regard, the dnRAR $\alpha$  mouse model will be a useful tool to study further the role of retinoids and their receptors in wound healing responses. Under normal vitamin A status, adult dnRAR $\alpha$  mice appear to have normal epidermal structure and cellularity. Presumably, basal levels of HB-EGF and other retinoid-insensitive growth factors (Figure 5) are sufficient to maintain epidermal thickness and functions that require only a low rate of cell proliferation in these transgenic mice in the laboratory environment. Alternatively, the unaffected retinoid signaling pathway in basal keratinocytes may be sufficient to support a low rate of homeostatic growth required for maintaining a normal epidermis. Targeted mutation or overexpression of the HB-EGF gene in suprabasal keratinocytes will be required to ascertain whether HB-EGF is the only major mediator in spatial regulation of epidermal growth by retinoid receptors. Our finding evokes the possibility that spatial regulation of HB-EGF-mediated cell growth by retinoid receptors may occur in other tissues during embryo development and/or in adulthood.

**The side effect, skin peeling, observed during retinoid therapy of skin disorders may not be eliminated by designing synthetic RAR-selective agonists**

Retinoids such as tRA are used widely in the clinical therapy of skin disorders such as acne vulgaris, psoriasis and photoaging (Craven and Griffiths, 1996). One of the major side effects caused by retinoids is skin peeling/flaking, due to an accelerated shedding of epidermal cells. Our findings suggest that this event is initiated by early induction of suprabasal HB-EGF followed by basal cell hyperproliferation. This ultimately causes a faster epidermal cell turnover, resulting in epidermal cell shedding/skin peeling. As retinoid induction of HB-EGF is mediated by RARs, design of new synthetic retinoids selective for

RARs may not eliminate this side effect since the receptors that mediate the side effects are probably the same as those mediating the therapeutic effects of retinoids.

## Materials and methods

### Construction of transgene K10-dnRAR $\alpha$

To construct K10-dnRAR $\alpha$ , a 1.5 kb *EcoRI*–*Bam*HI fragment from pSG5-mRAR $\alpha$ T403 (Durand *et al.*, 1994), which contains dnRXR $\alpha$  cDNA, was inserted into polycloning sites in pXJ81L between *EcoRI* and *Bam*HI sites, resulting in pXJ81L-mRAR $\alpha$ T403.

### Preparation of transgenic mice

K10-dnRAR $\alpha$  (7.2 kb) was liberated from plasmid vectors pXJ81L-mRAR $\alpha$ T403 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 F<sub>2</sub> hybrid zygotes from C57BL/6J $\times$ SJL/J parents (the Jackson Laboratory) at a concentration of 2–3 ng/ $\mu$ l as previously described (Feng *et al.*, 1997). After overnight incubation, the eggs which survived to the two-cell stage were transferred to day 0.5 post-coitum pseudo-pregnant CD-1 females (Charles River). Founder transgenic mice were mated to C57BL/6J mice to give F<sub>1</sub> offspring. F<sub>2-4</sub> offspring were produced by crossing F<sub>1</sub>–F<sub>3</sub> transgenic mice with C57BL/6J mice. The microinjection was carried out by the Transgenic Animal Model Core in the University of Michigan's Biomedical Research Core Facilities. Adult mice were fed with laboratory rodent diet #5001 (PMI Feeds Inc.). All procedures involving 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.

### Preparation of cDNA probes

Mouse CRABP II and  $\beta$ -actin cDNA probes were described previously. To prepare mouse EGFR (DDBJ/EMBL/GenBank accession No. X78987, positions 60–380), TGF- $\alpha$  (DDBJ/EMBL/GenBank accession No. M92420, positions 6–485), HB-EGF (DDBJ/EMBL/GenBank accession No. L07264, positions 226–570) and amphiregulin (DDBJ/EMBL/GenBank accession No. L41352, positions 76–520) cDNA, mouse epidermal RNA was reverse-transcribed and amplified using an RT-PCR kit (Stratagene) and a pair of corresponding primers of 30 bp. The resulting DNA fragments were subcloned into the PCR II vector (Invitrogen) and verified by sequencing. For Northern blotting, cDNA probes were labeled with [ $\alpha$ -<sup>32</sup>P]dCTP (3000 Ci/mmol) (DuPont-NEN) using a random priming kit (Life Technologies). For *in situ* hybridization experiments, cDNA containing the entire coding region of mouse HB-EGF (DDBJ/EMBL/GenBank accession No. L07264, positions 211–860) was cloned by RT-PCR.

### PCR genotyping, Northern and Southern blotting, gel mobility supershift and histological analyses

These analyses were performed as described previously (Feng *et al.*, 1997).

### Western blotting analysis of EGFR protein in mouse epidermis

After topical retinoid treatment, epidermal biopsies were taken as described previously (Feng *et al.*, 1997) and immediately frozen in liquid nitrogen. Epidermal whole cell extracts were prepared and analyzed by Western blotting according to Fisher *et al.* (1998). EGFR was analyzed using a rabbit IgG specific to its C-terminus (Santa Cruz Biotechnology Inc., Santa Cruz, CA) and visualized using the ECL system (Amersham Life Science Inc., Arlington Heights, IL). A retinoid-insensitive control protein,  $\beta$ -actin, was measured subsequently using a mouse monoclonal antibody specific to its N-terminus (Sigma).

### In situ hybridization analysis

To prepare sense and antisense probes, the PCR II vector containing the full-length HB-EGF cDNA was linearized by digestion with *Hind*III or *NotI*, respectively. Digoxigenin-UTP-labeled sense and antisense RNA probes were prepared from these templates using T7 or Sp6 RNA polymerases, respectively, and the DIG RNA labeling kit (Boehringer Mannheim). Punch biopsies (2 mm) were taken from mouse dorsal skin and embedded in an OCT compound, Tissue-Tek (Miles Laboratories). Frozen sections (7  $\mu$ m) were prepared from the specimens using a

cryostat and subjected to an *in situ* hybridization process as previously described (Kang *et al.*, 1995). RNA hybridization signals were revealed by staining with alkaline phosphatase-conjugated anti-digoxigenin antibodies.

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