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Nuclear receptors for retinoic acid and thyroid hormone regulate transcription of keratin genes

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In the epidermis, retinoids regulate the expression of keratins, the intermediate filament proteins of epithelial cells. We have cloned the 5′ regulatory regions of four human epidermal keratin genes, K#5, K#6, K#10, and K#14, and engineered constructs in which these regions drive the expression of the CAT reporter gene. By co-transfecting the constructs into epithelial cells along with the vectors expressing nuclear receptors for retinoic acid (RA) and thyroid hormone, we have demonstrated that the receptors can suppress the promoters of keratin genes. The suppression is ligand dependent; it is evident both in established cell lines and in primary cultures of epithelial cells. The three RA receptors have similar effects on keratin gene transcription. Our data indicate that the nuclear receptors for RA and thyroid hormone regulate keratin synthesis by binding to negative recognition elements in the upstream DNA sequences of the keratin genes. RA thus has a twofold effect on epidermal keratin expression: *qualitatively,* it regulates the regulators that effect the switch from basal cell–specific keratins to differentiation-specific ones; and *quantitatively,* it determines the level of keratin synthesis within the cell by direct interaction of its receptors with the keratin gene promoters.

Introduction

The profound effects of vitamin A on epidermis have been known for over 60 years (Frazier and Hu, 1931). Both in vivo and in vitro, retinoic acid (RA) deficiency causes hyperkeratosis and promotes differentiation of epidermal keratinocytes, whereas an excess of RA prevents keratinization and keeps keratinocytes in a basal layer–like phenotype (Fuchs and Green, 1981; Gilfix and Eckert, 1985; Nagae *et al.,* 1987; Asselineau *et al.,* 1989). RA and other retinoids strongly influence both epidermal morphogenesis and keratin gene expression in epidermal keratinocytes and other stratified epithelia (Fuchs and Green, 1981; Tseng *et al.,* 1984); Asselineau *et al.,* 1989). Retinoids are currently used in the treatment of a large number of epidermal disorders ranging from ichthyoses to wrinkles (Elias, 1986; Weiss *et al.,* 1988).

RA is a potent regulator of tissue morphology during development and differentiation (reviewed by Roberts and Sporn, 1984). RA presumably acts by initiating a cascade of events in which the early regulators effect the early morphological changes and induce the next class of regulators, which continue the process (La Rosa and Gudas, 1988; Lüscher *et al.,* 1989; Collins *et al.,* 1990). But, in addition, RA can control directly the transcription of differentiation-specific genes (Smits *et al.,* 1987; Bedo *et al.,* 1989). Transcription is regulated by the action of nuclear receptors, proteins that can bind both to specific DNA-recognition elements and to their respective ligands, in this case RA (reviewed in Evans, 1988; Green and Chambon, 1988; Nunez, 1989).

The RA nuclear receptor and the thyroid hormone (T3) nuclear receptor can recognize the same recognition element (RE) (Umesono *et al.,* 1988), and, in the presence of their ligands, each can increase initiation of transcription (effects of T3 were reviewed by Samuels *et al.,* 1988). Without its ligand, the T3 receptor, but not the RA receptor, can suppress transcription when bound to its RE (Damm *et al.,* 1989; Graupner *et al.,* 1989) or stimulate it in certain cell types (Forman *et al.,* 1988, 1989). Whereas the effect of T3 on mammalian keratin expression is not known, T3 affects keratin gene expression in *Xenopus laevis* by initiating a cascade of events

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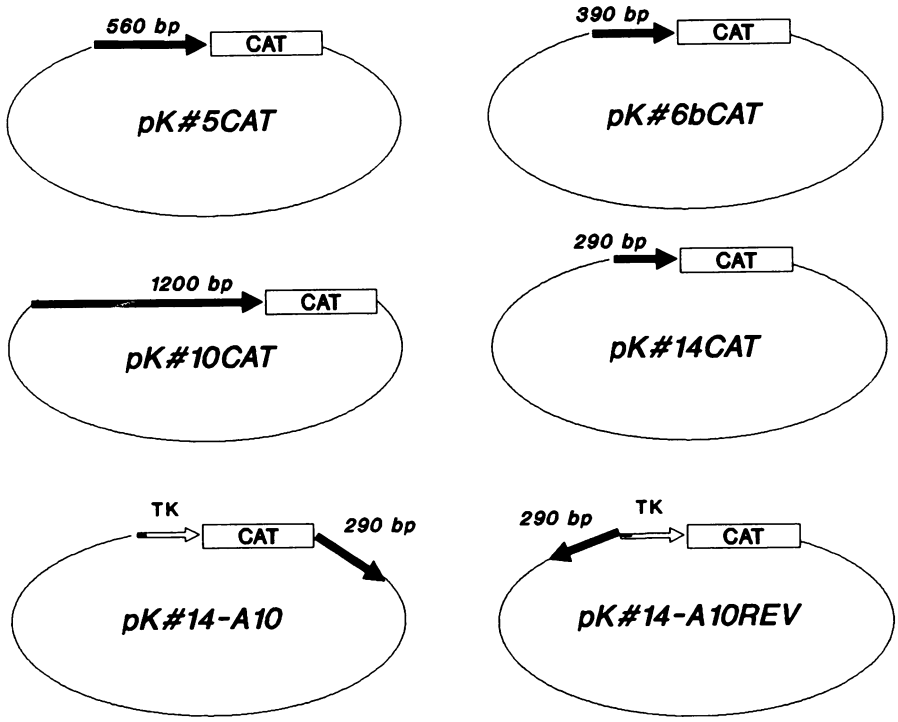

***Figure 1.* Structures of the keratin promoter-reporter gene constructs.** The filled-in arrows represent the promoter DNAs drawn to scale, with the direction of transcription indicated. The lengths of the DNAs extend upstream from the translation start sites for the indicated number of base pairs (bp). The boxes represent the reporter CAT gene and the ellipses the vector sequences. Open arrows represent the enhancerless promoter region of the HSV thymidine kinase gene (Hawley-Nelson *et al.*, 1988).

that leads to a high level of epidermal keratin production during development (Mathisen and Miller, 1989). T3 alters cornification and lipogenesis in the human epidermis, which contains the T3 receptor (Holt and Marks, 1977; Holt, 1978). Epidermal changes accompany hypothyroidism in ~90% of patients (Rosenberg *et al.*, 1986). T3 deficiency induces transglutaminase, which increases the production of cornified envelopes. At the same time, plasminogen activator levels are reduced, which inhibits desquamation (Iseroff *et al.*, 1989).

The effects of RA on morphogenesis and keratin gene expression in epidermal keratinocytes are particularly interesting, because it has been shown that the skin has a specific receptor for RA (Krust *et al.*, 1989; Zelent *et al.*, 1989). Retinoids regulate keratin gene expression at the mRNA level (Fuchs and Green, 1981; Eckert and Green, 1984). They suppress the expression of the basal cell–specific keratins K#5 and K#14 and the differentiation-specific keratins K#1 and K#10, as well as the hyperproliferation-specific keratins K#6 and K#16. The synthesis of keratins K#13 and K#19 seems to be induced (Kopan *et al.*, 1987). The molecular effects of RA on keratin expression are not defined: RA may control the keratin expression indirectly by regulating the differentiation process, or it may directly regulate the transcription of keratin genes *via* its nuclear receptors. An indirect mechanism has been implied for regulating simple epithelia-specific keratins K#7, K#8, and K#18 in HeLa cells (Stellmach and Fuchs, 1990); however, HeLa cells have very low levels of RA receptor. Indirect mechanisms seem to be operating in the F9 teratocarcinoma cells, in which keratins K#8 and K#18 are synthesized 4–5 d after induction of differentiation with RA (Grover *et al.*, 1983).

To determine the nature of regulation of expression of stratified epithelia-specific keratins, we have co-transfected vectors that express the nuclear receptors for RA and T3, with constructs in which regulatory regions of K#5, K#6, K#10, and K#14 keratin genes drive expression of the reporter CAT gene. We have found that RA and T3 directly affect the transcription of these keratin genes *via* the action of their nuclear receptors.

Results

The upstream regulatory regions from the functional genes of keratins K#5, K#6, and K#10, and of the K#14 pseudogene, were shown to act as promoters with tissue specificity: they were active in epithelial cells, but not in non-

epithelial cells. Furthermore, whereas K#14 DNA initiated transcription in all epithelial cells, including HeLa, the K#5, K#6, and K#10 promoters were functional only in primary cultures of cells derived from stratified epithelial tissues, but not in simple epithelial cells such as HeLa or mesothelial cells (Jiang *et al.*, 1990; Jiang, unpublished observations).

RA and T3 regulate four epidermal keratin promoters

To test the effects of RA and T3 on the control of expression of epidermal keratins, the constructs in which upstream sequences of functional genes encoding keratins K#5, K#6, K#10, and K#14 drive expression of the CAT reporter gene (Figure 1) were introduced into the primary cultures of rabbit corneal epithelial cells by co-transfection with the RA and T3 receptor expression vectors. All four keratin promoters were suppressed by both T3 and RA receptors in the presence of their ligands (Figure 2A). The relative activities of the four promoters vary in these cells, and, therefore, the levels of suppression are expressed as percentages of unsuppressed levels. The suppression ranges from 2-fold to >10-fold.

To determine whether the regulation can be generalized to different epithelial cell types, we have introduced all four keratin reporter constructs, pK#5CAT, pK#6CAT, pK#10CAT, and pK#14CAT, into primary cultures of rabbit esophageal epithelial cells (Figure 2B). All four constructs were regulated similarly by the RA receptor and the T3 receptor in esophageal cells as well, indicating that the regulation may be a general phenomenon present in all epithelial cells that contain the appropriate receptor.

Mode of action of nuclear receptors

To test the mode of action of nuclear receptors on keratin gene promoters, we chose the K#14 promoter because, of the four, it was the only functional one in HeLa cells. Although the K#14 DNA derives from a pseudogene, we have shown previously that it is functional and regulated in a cell-type–specific way (Jiang *et al.*, 1990). The levels of CAT were reduced by the addition of RA into the medium (Figure 3A), probably because of the relatively small amounts of the RA receptor in HeLa cells. In the absence of RA, co-transfection of the pK#14CAT plasmid with RA receptor plasmid did not affect the CAT levels. In the presence

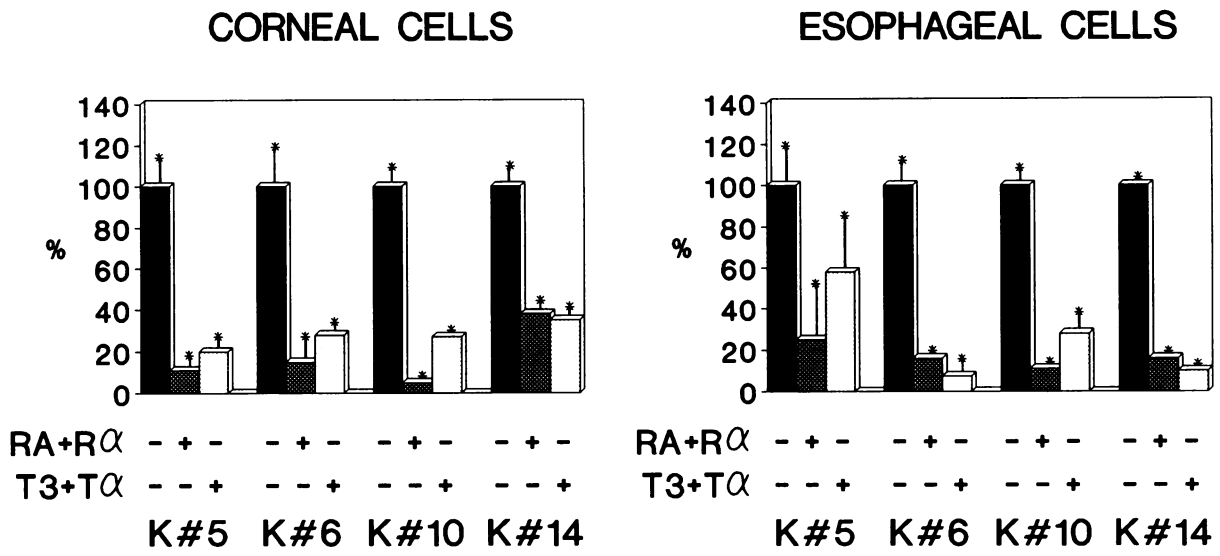

Figure 2. **Keratin genes K#5, K#6, K#10, and K#14 are regulated by the RA and T3 receptors.** The constructs in Figure 1 were co-transfected with pRSVZ internal control, and the CAT assay results are expressed corrected for the efficiency of transfection by normalizing the β-GAL levels. The asterisks on top of the bars represent the errors derived from multiple experiments. (A) Primary cultures of rabbit corneal cells. The relative activities of the four promoters in the RA- and T3-free medium were adjusted to the same height. The actual numbers are 2.52 units for K#5, 4.75 units for K#6, 0.89 units for K#10, and 7.60 units for K#14. For each keratin promoter, the relative ratios of suppression by the RA and T3 receptors are shown. (B) Primary cultures of rabbit esophageal cells. The actual activities of the four promoters in the esophageal cells in the T3- and RA-free medium are 0.12 units for K#5, 2.25 for K#6, 0.18 for K#10, and 3.36 for K#14.

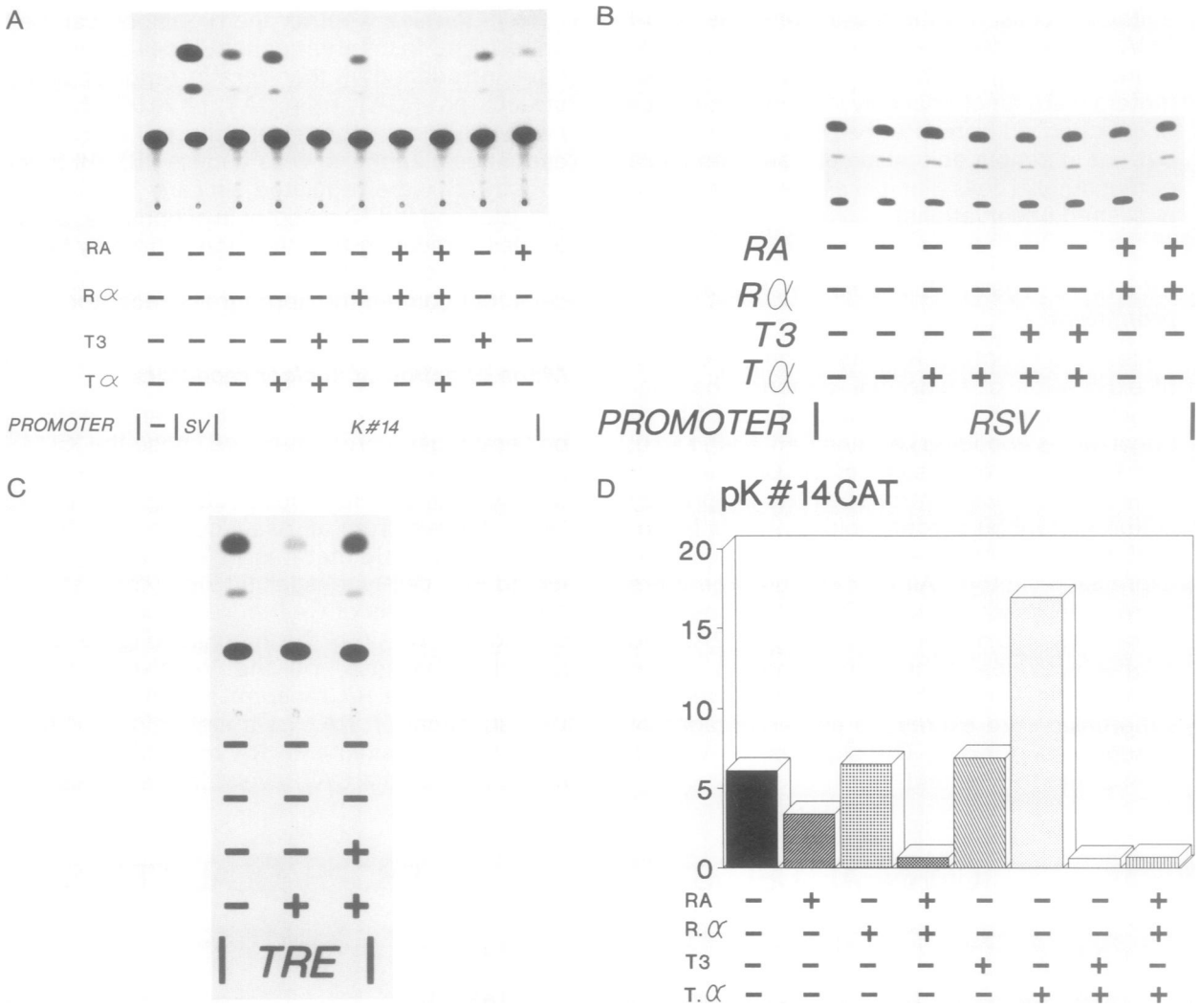

Figure 3. **Transcription of the K#14 promoter is regulated by the RA receptor and the T3 receptor.** (A) The parental control plasmid without any keratin DNA, pG-CAT, has no detectable levels of CAT transcription. pSV2CAT is a positive control plasmid. The plasmid pK#14CAT was cotransfected with combinations of nuclear receptor clones in the presence and absence of their ligands, as shown. (B) The control plasmid, pRSV-CAT, is not affected by the nuclear receptors in HeLa cells. (C) The plasmid containing a thyroid-hormone-responsive element, TRE, is regulated by the T3 receptor. (D) Quantitative presentation of data in Figure 3A. All numbers are normalized relative to the β-GAL internal control levels.

of RA, the nuclear receptor reduced the transcription of the pK#14CAT almost 90% (Figure 3A). No such effect was seen on the regulatory region of the Rous sarcoma virus in the control construct pRSV-CAT (Figure 3B). The effect of RA receptor is, therefore, specific for keratin gene and not an indirect result of alteration of HeLa cells' metabolism.

Co-transfection of pK#14CAT with the T3 receptor construct had an augmenting effect on the CAT levels. The thyroid hormone receptor, in the absence of its ligand, increased the pK#14CAT expression approximately threefold. In the presence of its ligand, T3 receptor is a strong repressor, similar to the RA receptor in the presence of RA (Figure 3A). The transcription of the control plasmid pRSV-CAT was unaffected (Figure 3B). The transcription of the control plasmid containing the thyroid recognition element (TRE) from the human growth hormone gene was suppressed by the T3 receptor in the absence of its ligand and increased in the presence of T3, as expected (Figure 3C). The effects on TRE are opposite of those on pK#14CAT, showing the specificity of the down-regulation of keratin promoters by T3.

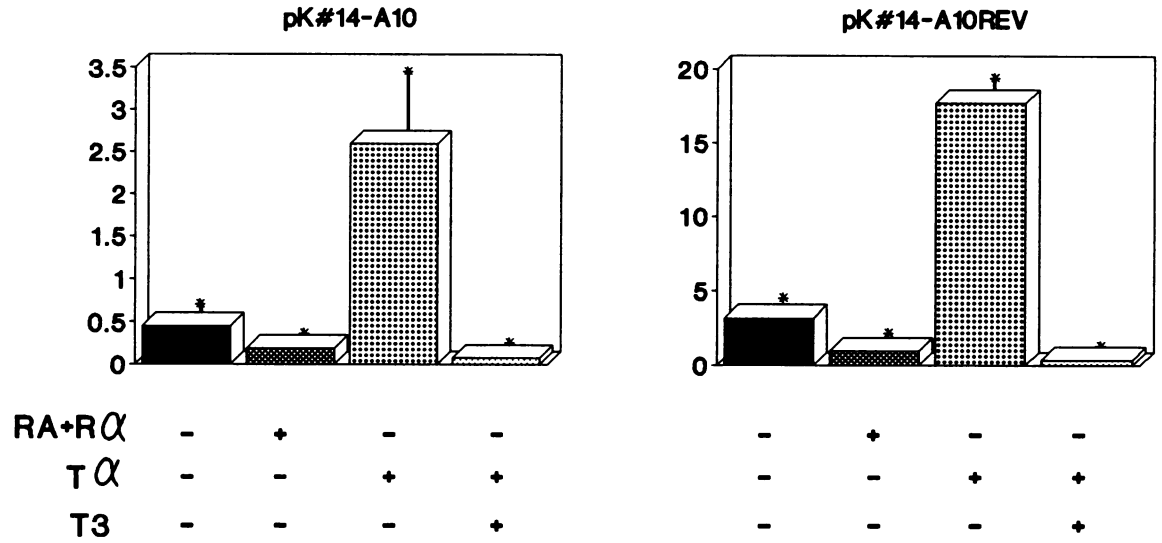

Figure 4. **The RA-responsive element of the K#14 gene is not position or orientation dependent.** T3 and RA regulate pK#14CAT transcription both in the wrong orientation (right panel) and when distal to the transcription start site (left panel) (see Figure 1). The parental control plasmid, pA10, has no detectable levels of CAT transcription. Note the scale difference of the two panels.

The RA receptor and the T3 receptor apparently can form heterodimers potentially modulating each other's function (Forman *et al.,* 1989; Glass *et al.,* 1989). Because the T3 receptor in the absence of T3 induces K#14 transcription, and the RA receptor in the presence of its ligand represses it, we co-transfected HeLa cells with both receptor constructs in the absence of T3, but in the presence of RA. The repression by RA and its receptor was clearly epistatic over the inducing effect of the T3 receptor (Figure 3, A and D). Although we have not attempted to quantitate the levels of the two receptors in the transfected cells, both DNAs were present at levels at least four times higher than those sufficient for the full effect on the keratin gene regulation (data not shown). It appears that the suppressing effect of the RA receptors in the presence of RA is stronger than the inducing effect of the T3 receptor without its ligand.

The positive regulatory effect of the nuclear receptors is enhancer-like, i.e., their function is not absolutely dependent on the position of the REs. To determine whether the negative regulatory effects of the nuclear receptors are also position independent, we engineered constructs in which the K#14 keratin gene regulatory sequence is in the reverse orientation in front of the enhancerless CAT gene from the pA10 plasmid (Hawley-Nelson *et al.,* 1988) (pK#14-A10REV in Figure 4), or in the proper orientation but in a more distal position, downstream from the CAT gene (pK#14-A10 in Figure 1). Each of the two constructs was co-transfected into HeLa cells with the receptor constructs. The enhancer properties of the K#14 DNA segment are stronger in the more proximal position, but the regulatory effects of RA and T3 receptors were the same in both constructs (Figure 4), indicating that the negative regulation of keratin gene transcription by the nuclear receptors for RA and T3 is not position dependent.

All three RA receptors have similar effects

Three different RA receptors have been described in human and murine tissues: alpha, beta, and gamma (Petkovich *et al.,* 1987; Brand *et al.,* 1988; Krust *et al.,* 1989). The last one appears specific for skin (Krust *et al.,* 1989; Zelent *et al.,* 1989). We co-transfected the pK#14CAT plasmid with each of the three receptor genes into HeLa cells (Figure 5). In the presence of RA, all three receptors suppressed the CAT expression to similar extents. In the absence of RA, none of the three receptors had any effect on the CAT levels. When co-transfected with the T3 receptor construct in the presence of RA and absence of T3, all three equally suppressed transcription. In the absence of both RA and T3, the T3 receptor increased the CAT levels when co-transfected with each of the three RA receptors (Figure 5). Thus, the three receptors have very similar ef-

fects on keratin gene expression under all the conditions we examined.

Discussion

Molecular mechanisms of regulation

Our data demonstrate that the nuclear receptors for T3 and RA can directly suppress the transcription of epidermal keratin genes. This effect is not due to direct competition with RNA polymerase for the DNA sites in the promoters, or with other components of the transcription machinery (Levine and Manley, 1989), because the suppressive effect is just as evident whether the keratin DNA is placed in the opposite orientation or downstream of the transcription start. The molecular mechanism for negative regulation of keratin genes, in which the receptors compete for nuclear factors (Meyer *et al.,* 1989; Levine and Manley, 1989), is difficult to reconcile with the augmenting effects of the T3 receptor in the absence of T3.

The suppressive effect of the RA and T3 receptors on keratin gene expression may be similar to the suppressive effect of the glucocorticoid receptor, which depends on a different DNA site, a negative glucocorticoid recognition element, nGRE. The consensus sequence of the nGRE binding site could not be determined, but the suppressive effect is mediated through direct binding of the receptor to the DNA (Sakai *et al.,* 1988). Similar nREs may exist in the upstream DNAs of the keratin genes.

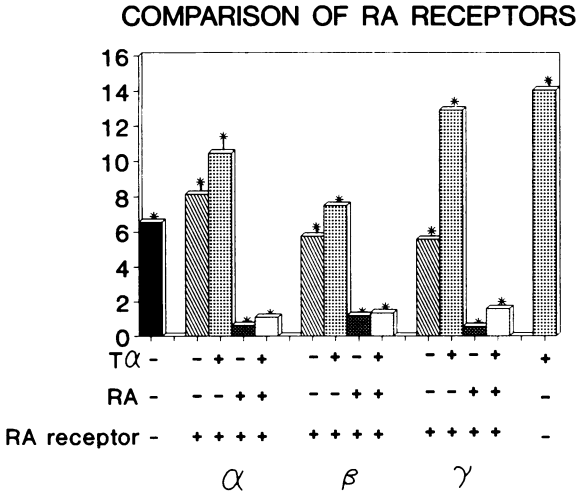

Figure 5. **The three RA receptors have similar effects on the pK#14CAT expression.** The error bars are shown as in Figure 2.

The T3 receptor in the presence of its ligand can induce, and in its absence can repress, transcription. The opposing effects of the T3 receptor were observed with the same TRE (Damm *et al.,* 1989). Similar results have been reported for prolactin gene expression (Forman *et al.,* 1988). The keratin genes are regulated by the T3 receptor in just the opposite way: they are repressed in the presence of T3 and induced in its absence. The K#14 promoter does not contain the perfect palindromic TRE sequence known to be recognized by the T3 and RA receptors, GGTCATGACC (Umesono *et al.,* 1988). The most similar site matches 7/10 base pairs, GGTGATGA*AA,* and is located −145 bp upstream from the translation initiation site. In this region the functional and the pseudogene sequences are identical. The T3 receptor effects on the K#14 promoter may be mediated by distinct positive and negative TREs. RA and T3 receptors, which can induce transcription from the same TREs, may suppress transcription from different nTREs.

The T3 nuclear receptor can affect transcription of the same gene differently in different cell types (Forman *et al.,* 1988), but the keratin genes seem to be similarly regulated by the T3 and RA receptors in the three cell types we tested—HeLa, rabbit corneal, and rabbit esophageal epithelial cells.

Regulation during epidermal differentiation

The epidermis is supplied with RA from serum through the basal lamina. The proximal, basal-layer cells, which experience the highest concentration of RA, express K#5 and K#14 keratins in relatively low amounts. The suprabasal cells switch to expressing K#1 and K#10 and, as they move away from the source of RA, become progressively fuller of keratins.

Although the molecular interactions of the RA receptor and keratin gene REs are yet to be elucidated, our data indicate that the expression of all epidermal keratin genes we have studied (K#5, K#6, K#10, and K#14) is suppressed by the receptor. We propose that the regulation of expression by the nuclear receptors for RA and T3 is a general phenomenon that applies to all epidermal keratin genes. Overall suppression of all keratins by RA in squamous cell carcinomas has been observed before (Kopan and Fuchs, 1989). Thus, the RA may not directly cause the switch in the expression of the basal vs. suprabasal keratins, as suggested previously (Kim *et al.,* 1984). We suggest that RA has two sepa-

rable effects on keratin synthesis in the epidermis: qualitative and quantitative. Qualitatively, it influences the differentiation process of the epidermal keratinocytes, presumably by regulating the expression of secondary regulators of the different stages of the keratinocyte differentiation pathway (Fuchs and Green, 1981; Asselineau *et al.,* 1989); it also determines whether the basal layer specific keratins K#5 and K#14 or the differentiation specific keratins K#1 and K#10 are expressed. Quantitatively, it directly effects the level of expression of keratins (and probably other epidermal proteins) by the action of its receptor on the regulatory sites in their genes. The fact that RA influences epidermal metabolism both by directly affecting regulation of genes and by regulating the differentiation process has important consequences regarding the development of pharmacologically important retinoids for treatment of human epidermal disorders.

Materials and methods

DNA constructs

The clone containing the K#14 pseudogene promoter linked to the CAT gene has been described previously (Jiang *et al.,* 1990). Using PCR, we have constructed similar clones containing 560 bp of the promoter of the human K#5 gene (Lersch *et al.,* 1989), 390 bp of the K#6b gene (Tyner *et al.,* 1985), and 1200 bp of the human K#10 gene (Rieger and Franke, 1988). The details of their construction will be reported elsewhere (Jiang *et al.,* not-yet-published observations). We determined the sequences of all PCR-amplified DNAs using the dideoxy plasmid sequencing method (Chen and Seeburg, 1985). The control plasmid pRSV-CAT was a gift from C. Gorman. The clones containing the receptor genes, HEO, CAS, kRARjeO, RAR-αO, RAR-βO, and chic-c-erb-A (Tα) have also been described (Petkovich *et al.,* 1987; Brand *et al.,* 1988; Forman *et al.,* 1988; Krust *et al.,* 1989). The TRE-responsive element, TRE-3 × 3-tk-CAT, contains three copies of the palindrome TRE from the human growth hormone gene in front of the tk promoter (P. Chambon, unpublished observations). All DNA samples were purified by two CsCl-ethidium bromide equilibrium banding centrifugations.

Cell maintenance and growth

HeLa cells were maintained in Dulbecco-modified Eagle's medium (DMEM) supplemented with 10% calf serum (FCS). All media contained penicillin and streptomycin. Cells were grown at 37°C in a 5% CO_2 atmosphere. The primary cultures of rabbit corneal and esophageal epithelial cells were initiated as described before (Schermer *et al.,* 1986) and maintained in DMEM with 20% FCS and 5 ng/ml of hydrocortisone in 60-mm^2 dishes until they were ~60% confluent. The two epithelial cell types were grown in the presence of a 3T3 fibroblast feeder layer, as described (O'Guin *et al.,* 1987). They were transformed using the calcium phosphate precipitation procedure (Jiang *et al.,* 1990). After mitomycin treatment, fibroblasts do not express any CAT activity when transformed with functional CAT constructs (data not shown). T3 and RA (Sigma) were removed from the medium using a published procedure (Casanova *et al.,* 1985) and supplemented as needed at the levels of 10^{-9} and 10^{-7} M, respectively.

Cell transformation

We have generally followed the procedure of Chen and Okayama (1987). One day before transformation, cells were plated onto 100-mm plates at the optimal density that produces a near-confluent culture when the cells are harvested. The medium was changed 4 h before transformation. Into each dish, we added 5 μg of the CAT plasmid, 2 μg of the receptor expression vector plasmid, 2.5 μg pRSVZ reference plasmid, and a sufficient amount of carrier to bring the total to 20 μg of DNA. In separate experiments we have shown that this amount of receptor expression vector plasmid is at least fourfold higher than the saturating levels. Forty-eight hours after transfection, the cells were harvested by scraping and subjected to sonication in a 150-μl volume.

Primary cultures were transformed and assayed similarly except that they were not passaged before transfection. Usually 5 μg of CAT plasmid and 1.25 μg of pRSVZ control were used in corneal and esophageal cells, and each experiment was repeated in duplicate with primary cultures initiated from at least two different rabbits.

Enzyme assays

The β-GAL activity serves as the internal control to monitor transfection efficiency for each dish. It was assayed as described (Jiang *et al.,* 1990). The β-GAL activity was calculated according to the following formula

$$\frac{1000 \times OD_{420}}{\mu g \text{ pRSVZDNA}} \times \frac{150}{\mu l \text{ of extract used}} \times \frac{60}{\text{mins of reaction time}}$$

For CAT assays, 50 μl of each extract was heated to 65°C for 10 min, clarified by centrifugation for 10 min, and used as described by Gorman *et al.* (1982). After silica gel chromatography and autoradiography, the spots were excised from the thin-layer plates and counted. The conversion of chloramphenicol to its monoacetylated derivative was kept below 50%. The CAT activity is calculated according to the following formula

$$\frac{\text{cpm[Cm} - \text{OAc]}}{\text{cpm[CmOAc} + \text{Cm]}} \times \frac{150\mu l}{50\mu l} \times \frac{1}{\mu g[\text{CAT plasmid}]}$$

for a 1-h reaction. The numbers were normalized for transfection efficiency within a given cell type by calculating the ratio of CAT activity to β-GAL activity in each transfected plate.

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