Retinoic Acid Suppression of Loricrin Expression in Reconstituted Human Skin Cultured at the Liquid-Air Interface

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Retinoids have been shown to modulate the expression of proteins involved in epidermal differentiation. To examine this effect in an *in vitro* skin model, we evaluated the effect of retinoic acid on the expression of two cell envelope proteins, loricrin and involucrin, and an early marker of epidermal differentiation, keratin 1, in a reconstituted human skin equivalent cultured at the liquid-air interface. Retinoic acid, a known inhibitor of keratinization in monolayer and raft cultures, was evaluated for its ability to alter the expression and distribution of these markers of epidermal differentiation. Retinoic acid (10^{-6} M) suppressed loricrin expression in skin cultures as determined by both protein and mRNA analysis. In contrast, no inhibition of involucrin or K1 expression was observed at the protein level at the same retinoic acid concentration. However, some suppression of K1 mRNA transcription was observed in retinoic acid-treated cultures. These results demonstrate that in differentiating cultures of reconstituted human skin, loricrin expression is markedly inhibited by retinoids, K1 less so, and involucrin not at all.
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The corneocyte envelope is a chemically resistant structure located beneath the plasma membrane, which is formed by the crosslinking of protein precursors assembled in terminally differentiated epidermal keratinocytes [1–3]. A number of proteins have been shown to be incorporated in the envelope [4–9]; however, attention has centered on two of these precursors, loricrin [8–11] and involucrin [1,12–15]. Whereas both participate in the assembly of the complete envelope, they are differentially expressed. Loricrin is expressed at later stages of *in vivo* differentiation than is involucrin, as seen by its less extensive distribution and restriction to the upper epidermis (compare [8,9] with [14,15]). Involucrin is expressed in submerged cultures grown on plastic in both low- and high-calcium media [16] whereas loricrin is expressed only in high-calcium media [10,11]. With the addition of retinoids, loricrin expression is suppressed [10], whereas involucrin is not [15,17].

The culture of epidermal keratinocytes on fibroblast-contracted collagen gels at the liquid-air interface [18–21] provides the opportunity to study the mechanisms by which the expression of these two proteins is regulated in a system where cell interactions more closely resemble that of the developing epidermis. Therefore, in the present study we analyzed the expression of these cell envelope proteins in this three-dimensional culture system, and examined the effects of retinoids on the synthesis of these and other markers of epidermal differentiation.

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Abbreviations: K1, human keratin 1; K14, human keratin 14; RA, retinoic acid; SE, human skin equivalent cultured at the liquid-air interface.

MATERIALS AND METHODS

Keratinocyte Cultures Human keratinocytes were isolated from foreskin following an overnight incubation in 1% trypsin in versene (Sigma Chemical, St. Louis, MO). The cells were placed on feeder layers of mitomycin-C–treated Swiss 3T3 (American Type Culture Collection, Rockville, MD) fibroblasts. The cultures were maintained in an epidermal growth media (E-media) containing the high-glucose form of Dulbecco's Modified Eagle Media (DMEM) supplemented 3:1 with Ham's F-12 Nutrient Mixture (Gibco, Grand Island, NY), 5×10^{-10} M cholera toxin (Schwarz-Mann Biotech), 2 μg/ml hydrocortisone, 25 μg/ml insulin, 25 μg/ml transferrin (Sigma), and 1×10^{-10} M triiodothyronine [19].

Construction of the Skin Equivalent (SE) Skin equivalents were prepared using a variation of a technique established by Bell and coworkers [20,21] and modified by Kopan *et al* [19]. Briefly, normal human neonatal dermal fibroblasts at $3.3–4.7 \times 10^4$ cells/ml in 3 ml DMEM with 10% calf serum were mixed with 1 ml of 2.5 mg/ml rat tail collagen (Collaborative Research, Bedford, MA). The mixture was allowed to gel and contract in a sterile 35-mm petri dish [20]. After approximately 5 d, keratinocytes were added to the surface of the dermal equivalent. The resulting SEs were grown at 37°C and 7% CO_2 [19,21], submerged in E-media. Cultures remained submerged for approximately 1 week to allow for epidermal attachment and monolayer formation. At this time they were placed on a steel mesh grid at the liquid-air interface. From this point, untreated cultures were fed every other day with E-media, while treated cultures were fed on the same schedule with varying concentrations of all-trans retinoic acid (Sigma), which was dissolved in ethanol, in the media [19]. The final concentrations of ethanol in all cultures was 1%. Solutions were prepared and dosing was performed under yellow-light conditions. Retinoic acid (RA) was then refrigerated and stored under nitrogen in foil-covered amber bottles to protect from the light. The equivalents were harvested on day 10 of treatment and frozen in liquid nitrogen.

Immunohistochemical Localization Tissues were embedded in Histo Prep embedding compound (Fisher, Springfield, NJ). Four-micron sections

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of both skin equivalents and foreskin were cut on a Minotome cryostat, mounted on gelatin-coated slides, air dried, and subsequently fixed in acetone (−20°C) for 5 min. Sections were stained by standard indirect immunochemical techniques [22]. Control sections were incubated with the appropriate nonimmune antiserum and non-specific staining was not observed. The primary antisera used were rabbit anti-human involucrin (a total of four antibodies were tested, one from Biomedical Technologies Inc. [BTI], two graciously provided by Dr. Marcia Simon [23], and one provided by Dr. Scott Thatcher [24]; only the BTI results are shown), rabbit or guinea pig anti-human keratin 1 (K1) [25], and rabbit anti-loricrin [8].

Sections visualized by immunofluorescence were hydrated in phosphate-buffered saline followed by sequential incubation in 12% bovine serum albumin in 0.01 M Tris-buffered saline, normal blocking serum, primary antibody, and finally, the fluorescein-conjugated secondary antibody (Sigma or Dako-Patts). For immunoenzyme staining, the procedure was similar except the last incubation is replaced by a sequential incubation in biotinylated secondary antibody (Vector, Burlingame, CA or BTI; not shown) followed by the avidin-biotin-peroxidase complex (Vector or BTI; not shown). Antibody binding was then visualized using 3,3′-diaminobenzidine (Sigma). The sections were then counterstained in hematoxylin (Sigma) and mounted. All incubations were done in a humidity chamber.

RNA Isolation Total RNA was extracted from the skin equivalents (two per RA concentration) with RNAzol B (Biotecx Laboratories, Inc., Houston, TX) based on a previously described method [26]. The RNA was reprecipitated in the presence of 0.2 M NaCl with 2 vol of ethanol for 1 h at −20°C, then washed twice with 75% ethanol and resuspended in 40 μl of 1 mM ethylenediamine tetraacetate in diethylpyrocarbonate-treated water and stored at −70°C.

cDNA Synthesis Five micrograms of total RNA in 8 μl of water was heated to 65°C for 5 min, chilled on ice, and then added to a master mix containing 2 μl 10 × reverse transcriptase buffer (1 × RT) buffer is 50 mM Tris-HCl, pH 8.15, at 41°C; 40 mM KCl, 6 mM $MgCl_2$, 10 mM dithiothreitol, and 20 U of RNasin (Promega, Madison, WI), 2 μl of each 10 mM deoxynucleotide triphosphate (dNTP) and 1 μl of 10 μg/μl random hexamer primers [pd(N)$_6$, Pharmacia, LKB Biotechnology, Inc., Piscataway, NJ]. One microliter of 20 units/μl avian myeloblastosis virus reverse transcriptase (Life Sciences, St. Petersburg, FL) was added to give a final volume of 20 μl was incubated at 41°C for 90 min. The resultant cDNA was diluted 1 in 50 with water and stored at −20°C.

Polymerase Chain Reaction (PCR) Amplification Oligonucleotides for PCR were designed to yield a product size of the order 100–400 base pair (bp) and to span an intron to safeguard against the possibility of spurious amplification of contaminating genomic DNAs. The sequences of the 5′ and 3′ oligonucleotides are shown for their respective mRNA species, as follows: for human keratin 14, 5′-TTCTCACAGCCACAGTGGAC-3′ and 5′-CATTGACATCTCCACCCACC-3′; for human involucrin, 5′-TGCCTCAGCCTTACTGTGAG-3′ and 5′-GCAGTCCCTTTACAG-CAGTC-3′; for human loricrin, 5′-TCACTCACCCTTCCTGGTGC-3′ and 5′-CACCGCCGCCAGAGGTCTTC-3′; and for human keratin 1, 5′-ATAGCCCAGAAGAGCAAAGC-3′ and 5′-TCGGGGCACATTCT-CCAGAC-3′. Based on the published cDNA sequences, these primers were expected to generate fragments of 281 bp for keratin 14 [27], 251 bp for involucrin [28], 116 bp for loricrin [9], and 418 bp for keratin 1 [29].

The PCR reaction of 100 μl consisted of 10 μl 10 × PCR Buffer (Perkin Elmer Cetus, Norwalk, CT), 2 μl each 10 mM dNTP, 1 μl diluted cDNA, 1 μl of 5′ primer at 200 ng/μl, and 1 μl of 3′ primer at 200 ng/μl, 78 μl H_2O, and 1 μl (5 units/μl) of *Thermus aquaticus* (Taq) DNA polymerase (Perkin Elmer Cetus). Reactions were performed in a DNA thermal cycler according to the manufacturer's instructions. Samples were heated to 95°C for 5 min, then cooled to 50°C for 2-min annealing and the second strand extended at 72°C for 10 min. Subsequently, 30 cycles of amplification were carried out using a step program (denaturation, 95°C for 30 sec, annealing, 60°C for 1 min, and extension, 72°C for 1 min). The final extension was at 72°C for 15 min.

RESULTS

Histology The SE (Fig 1*A*) exhibited a similar morphology when compared to normal human foreskin. Clearly distinguishable stratified epidermal cell layers were formed in the SE, including a stratum basalis, spinosum, granulosum, and corneum-like layers. Differences between the two systems are also evident. Specifically, the model has a parakeratotic stratum corneum and lacks dermal papillae, vasculature, and nerves.

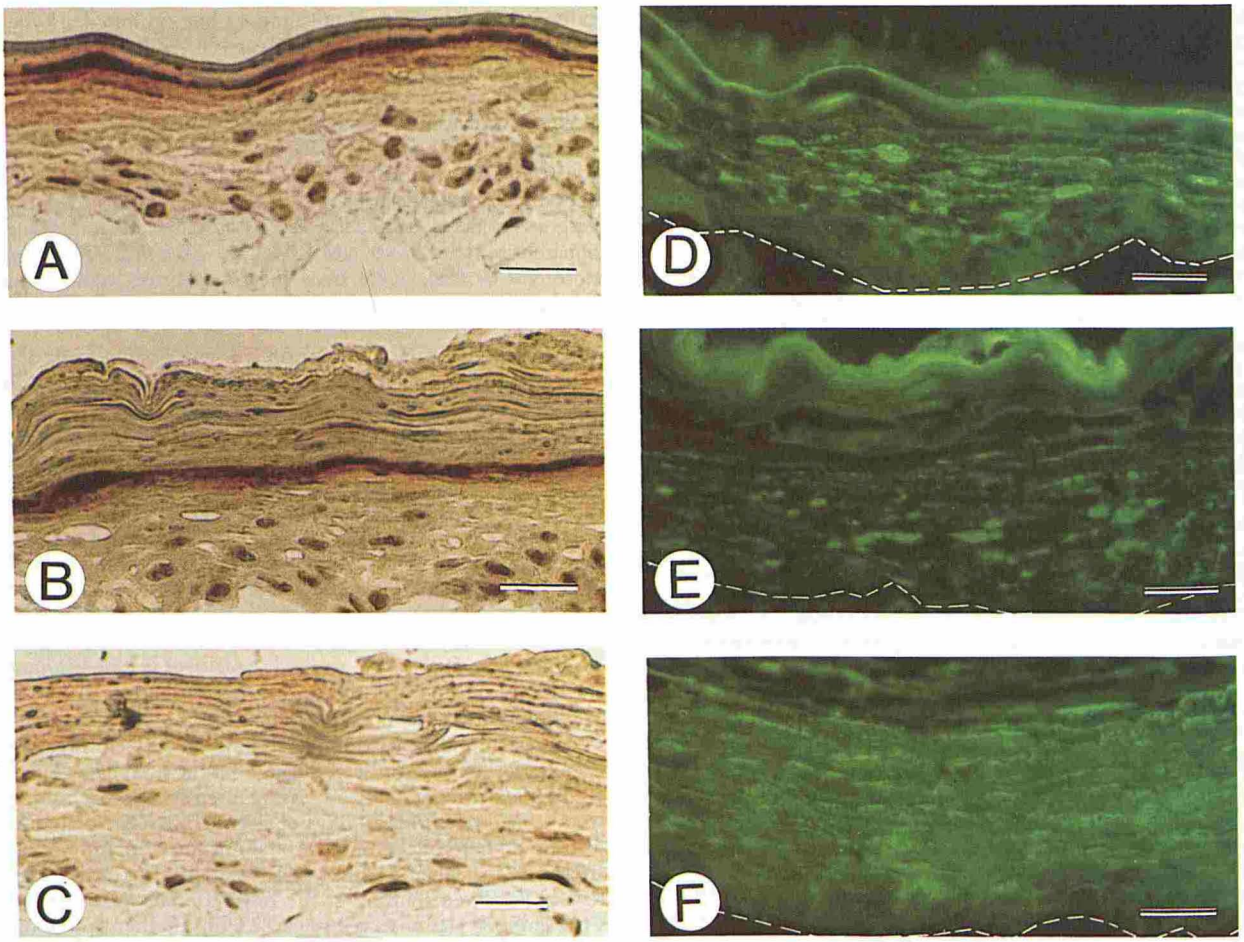
SE cultures treated with RA at 10^{-7} and 10^{-6} M (Fig 1*B,C*) retained their keratinized morphology but exhibited a hyperplastic response that was most prominent at 10^{-6} M RA. The epidermis of cultures treated with RA showed a dose-dependent decrease in intercellular adhesion resulting in less cell-to-cell contact, as has been reported previously [19]. The loss of intercellular adhesion was particularly marked at 10^{-6} M RA. Cultures grown in the presence of 10^{-5} M RA did not develop a keratinizing epidermis (data not shown), but instead varied in thickness from multiple cell layers to a single very darkly stained layer that appeared necrotic. Due to the apparent toxicity of 10^{-5} M RA on the SE cultures, only nontoxic doses ($\leq 10^{-6}$ M) were used for comparisons of differentiation markers.

Immunolocalization Loricrin is a major component of the keratinocyte cell envelope and is expressed late in epidermal differentiation [8]. In the untreated skin equivalent (Fig 1*A*) staining was restricted to the epidermis, specifically from the upper stratum spinosum to the stratum corneum. This localization is comparable to that seen in human epidermis *in vivo* [9]. Loricrin was still detectable at 10^{-7} M RA (Fig 1*B*); however, the staining intensity was markedly reduced at 10^{-6} M RA (Fig 1*C*). A similar but less intense pattern of expression was obtained when using an antibody directed against the N-terminal portion of loricrin (DR Roop, unpublished data) (data not shown). The basis for the staining of the epidermal nuclei in the skin equivalent is not known; however, it appears to be specific to the C-terminal antibody because it was not present when staining with the N-terminal–directed antibody.

Involucrin is a corneocyte envelope protein that is expressed early in epidermal differentiation [1]. In untreated SE cultures (Fig 1*D*), involucrin staining was seen throughout the suprabasal layers of the epidermis, with gradually increasing intensity approaching the stratum corneum. Unlike *in vivo* epidermis, involucrin staining was distributed heterogeneously throughout the cytoplasm rather than at the cell periphery. This pattern was specific to the SE because foreskin showed staining at the cell periphery (data not shown). This staining was observed regardless of whether immunoperoxidase or immunofluorescence methods of detection were used and occurred with all involucrin antibodies listed in the *Materials and Methods* section. Skin equivalent cultures reach a proliferative rate in the range of normal skin after approximately 16 d at the liquid-air interface. Cytoplasmic staining of involucrin may be attributed to the hyperproliferative state of our short-term cultures (10 d) of SEs, where it could be speculated that a change towards peripheral staining may occur in cultures grown longer at the liquid-air interface ([30,31], NL Parenteau personal communication). Involucrin (Fig 1*F*) could be detected by antibodies at a RA concentration of 10^{-6} M, in contrast to loricrin, which was inhibited at this concentration. The addition of RA produced no change in the distribution of involucrin (Fig 1*F*) and similar results were obtained regardless of the involucrin antibody used (data not shown).

Keratin 1, an early marker of differentiating epidermal cells, was expressed suprabasally in untreated SE (Fig 1*G*) cultures. K1 staining was also evident at all doses of RA treatment; however, the distribution appeared to be restricted to the uppermost layers of the SEs (Fig 1*H,I*).

RNA Analysis The relative abundance of RNA transcripts for K1, K14, involucrin, and loricrin was determined by reverse transcriptase/PCR [32]. Loricrin mRNA was undetectable at RA concentrations of 10^{-5} and 10^{-6} M (Fig 2). Some suppression of loricrin mRNA transcription was also evident at a RA concentration of 10^{-7} M. K1 transcription appeared partially suppressed at RA concentrations of 10^{-5} and 10^{-6} M. In contrast, gene expression for both the basally expressed keratin K14 and suprabasally expressed involucrin were refractory to the presence of RA. Thus an argument can be made that the RA effect is specific for a subset of keratinocyte genes and not a consequence of a global effect due to cell poisoning and/or tissue necrosis. The suppression of K1 [25,33] and loricrin [10] expression by RA has previously been observed in submerged cultures.



DISCUSSION

Skin equivalent culture systems have the advantage over monolayer keratinocyte cultures in that they not only express all of the markers of the epidermis but also mimic the architecture of the epidermis. In the present study we demonstrate the expression of both early and late markers of differentiation in an SE model employing a contracting dermal matrix. The expression of these markers in this system was similar to that of *in vivo* epidermis with respect to both onset and localization. The early marker, K1, was detected throughout the suprabasal layers. Involucrin expression had a similar distribution in these cultures and preceded loricrin expression, which was restricted to the upper layers.

Having established the expression of these markers in our SE model, we tested the effect of RA on their expression in this model. Interestingly, the expression of the two envelope proteins was differentially affected by RA in these SEs. Involucrin expression (Fig 1*F*) was not inhibited at retinoid concentrations shown to inhibit the expression of loricrin (Fig 1*C*) in developing SEs. The lack of loricrin expression was not due to a masking of the C-terminal loricrin epitope in response to RA because inhibition was also seen with an antibody to the N-terminal region (data not shown). Moreover, mRNA analysis demonstrated that loricrin transcripts were not present in cultures treated with 10^{-6} M RA. An apparent reduction in loricrin mRNA was already observed at a RA concentration of 10^{-7} M, in contrast to involucrin transcripts, which were present in all RA concentrations tested. In comparison, SE cultures treated with 10^{-6} M RA also exhibited a partial reduction in K1 mRNA levels and no reduction in mRNA levels for K14. Although PCR amplification is only semiquantitative, these data are consistent with the previous report on the expression of K14 and K1 at the protein level [19]. Therefore, the separate regulation of loricrin and involucrin in skin equivalents appears to occur at the mRNA level.

In the course of these studies, similar results were published [34] showing loricrin suppression in skin cultures treated with RA. In addition to these findings, we have demonstrated in our model the expression of K1 in conjunction with a morphologically normal-appearing epidermis (Fig 1*I*) at concentrations of RA that inhibit loricrin expression (Fig 1*C*). In our hands at 10^{-6} M RA, there is an apparent reduction, but not elimination, in the amounts of K1 mRNA, which may explain the more restricted staining for K1 to the upper suprabasal layers. Complete inhibition of K1 expression by RA in SEs has been associated with loss of the keratinizing phenotype in several previous studies [18,19,34,35]. However, in our studies K1 is expressed at both the mRNA (Fig 2) and protein levels (Fig 1*I*) at 10^{-6} M RA. The expression of K1 in our cultures at 10^{-6} M RA may be associated with the presence of a keratinized phenotype at this concentration. This is consistent with the acute response of human epidermis *in vivo* to topical RA treatment, where the keratinized phenotype was maintained, as was K1 expression, but with an inhibition of loricrin expression [36]. Differential expression of loricrin and K1 has also been reported in response to phorbol ester treatment [37] and changes in calcium concentration in cultured mouse keratinocytes [11].

We have determined several differences in our culture conditions, compared to those previously reported [19,34], which could account for the difference in K1 sensitivity to RA at 10^{-6} M. Considering the substantial increase in added RA over the concentration of RA in serum, it is unlikely that variations in serum RA levels in these different studies could account for the differences observed in K1 expression. Because there are some differences in the media used in our study and that of Magnaldo *et al* [34], it is possible that certain

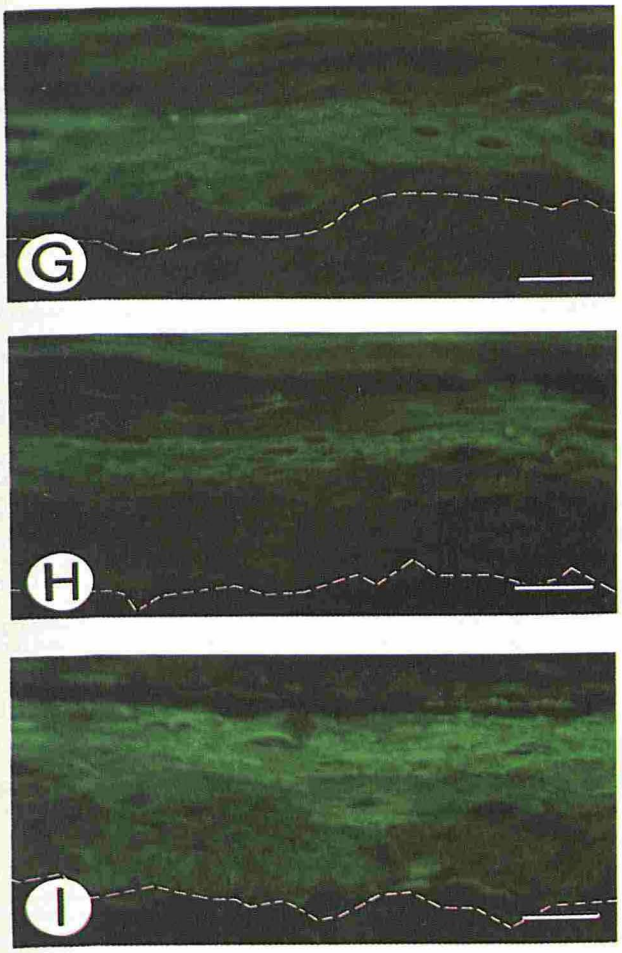


Figure 1. Immunolocalization of loricrin, involucrin, and K1. The distribution of the envelope proteins loricrin *(A–C)* and involucrin *(D–F)* and the keratin K1 (G-I) is shown for skin equivalents treated with 1% ethanol *(A,D,G)*, 10^{-7} M RA in 1% ethanol *(B,E,H)*, or 10^{-6} M RA in 1% ethanol *(C,F,I)*. The procedure for immunoenzyme *(A–C)* and immunofluorescent *(D–I)* techniques is described in the *Materials and Methods*. *Bar*, 10 μM.

media or serum components may influence retinoid regulation of epidermal differentiation. In addition, our dermal matrix employs human skin fibroblasts, which differs from the previous reports using immortalized mouse fibroblasts [19,34]. If the dermis is responsible for making the retinoid available to the epidermis, cell origin may be a factor in RA activity by effecting the bioavailability of the retinoid. Alternatively, fibroblasts with different origins may have the potential to differentially influence the expression of keratinization markers in the epidermis of SEs [38].

The differential expression of the two envelope proteins loricrin and involucrin within the epidermis raises an interesting paradox concerning corneocyte envelope assembly. Skin equivalents that differentiate in the presence of 10^{-6} M RA exhibit an apparently cornified epithelium in the absence of loricrin, raising the possibility that cell envelopes are being formed without loricrin participation. Similar observations have been made in monolayer cultures that express involucrin [16] but not loricrin [10], yet will form an envelope-like structure [39]. However, these envelopes are less rigid than those formed *in vivo* [39]. It remains to be determined whether envelopes are being formed in the SE system. If this can be demonstrated, those formed in the presence or absence of RA may have different biophysical properties attributed to the absence of loricrin. Thus, the SE would make an appropriate model for studying the contribution of loricrin to envelope structure and its properties.

The results of this study further support the use of *in vitro* skin model cultures to analyze the effects of pharmacologic agents, such as retinoids, on human skin. Although these culture models are not identical replicas of the morphologic and biochemical status of normal skin, their use can provide significant insight into the molecular mechanisms that control epidermal differentiation.

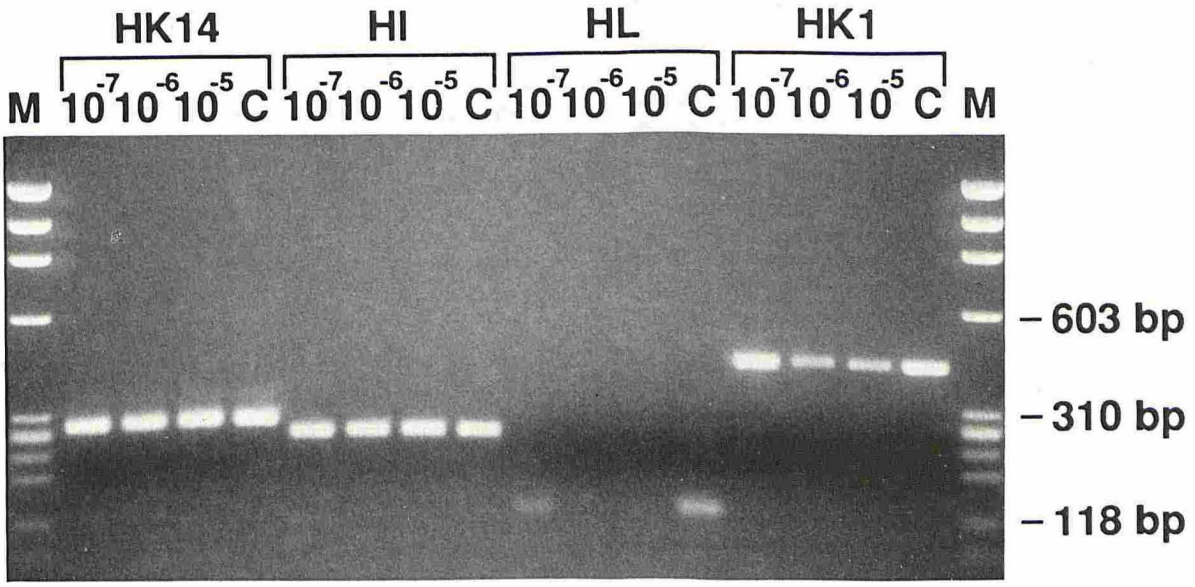


Figure 2. RNA transcript levels for retinoic acid–treated skin equivalents. Analysis of transcription by PCR showing the relative abundance of RNA transcripts for keratin 1 (HK1), keratin 14 (HK14), involucrin (HI), and loricrin (HL) in untreated and RA treated skin equivalents. Five microliters of each PCR sample (5% by volume) was electrophoresed on a 2% agarose gel and visualized with ethidium bromide. M, molecular size standards (Hae III digested ΦX174); C, control tissue/untreated skin equivalents; 10^{-5}, skin equivalents treated with 10^{-5} M RA; 10^{-6}, treated with 10^{-6} M RA; 10^{-7}, treated with 10^{-7} M RA. To test for the possibility of amplification of processed pseudogenes from contaminating DNA, RNA samples were directly subjected to 30 cycles of PCR using the K14-specific oligos and no products were detected (data not shown).

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