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Retinoid-mediated transcriptional regulation of keratin genes in human epidermal and squamous cell carcinoma cells

(keratin expression/retinoic acid/differentiation/transcriptional control/protein stability)

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ABSTRACT **Vitamin A and other retinoids profoundly inhibit morphological and biochemical features of epidermal differentiation *in vivo* and *in vitro*. To elucidate the molecular mechanisms underlying the differential expression of epidermal keratins and their regulation by retinoids, we examined retinoid-mediated changes in total protein expression, protein synthesis, mRNA expression, and transcription in cultured human keratinocytes and in squamous cell carcinoma (SCC-13) cells of epidermal origin. Our studies revealed that the epidermal keratins, K5, K6, K14, and K16, their mRNAs, and their transcripts were diminished relative to actin as a consequence of retinoic acid (RA) treatment. The effects were most pronounced in SCC-13 and were detected as early as 6 hr post-RA treatment, with enhancement over an additional 24–48 hr. Repression was also observed when 5′ upstream sequences of K14 or K5 genes were used to drive expression of a chloramphenicol acetyltransferase reporter gene in SCC-13 keratinocytes. Both cell types were found to express mRNAs for the RA receptors α and γ, which may be involved in the RA-mediated transcriptional changes in these cells. The rapid transcriptional changes in epidermal keratin genes were in striking contrast to the previously reported slow transcriptional changes in simple epithelial keratin genes.**

Retinoids have a profound effect on epithelial differentiation: they enhance morphological features characteristic of simple epithelia and inhibit terminal differentiation in epidermis and other stratified squamous epithelia (1, 2). As major structural proteins of epithelial cells and as markers linked to differentiation state and cell type, keratin expression patterns are often influenced by concentrations of retinoids that also modify differentiation (3–6). In F9 teratocarcinoma cells, retinoic acid (RA) enhances differentiation and expression of the simple epithelial keratins K8 and K18 (6, 7). In mesothelial and kidney epithelial cells, RA enhances expression of keratins K7 and K19 as well as K8 and K18 (4, 8). In epidermal cells, RA inhibits expression of differentiation-specific keratins K1 and K10 (3) and K6 and K16 (5), and to a lesser extent the basal cell keratins K5 and K14 (9, 10). For reasons that are presently obscure, the RA-mediated effects on epidermal keratin expression are even more pronounced on epidermal squamous cell carcinoma lines than they are on normal epidermal keratinocytes (10, 11).

Most RA-mediated changes in keratin expression are reflected at the level of their mRNAs (3–6). For simple epithelial cells, transcriptional control of keratin genes has been observed, although both mRNA and transcriptional changes are gradual, taking place over 48–96 hr post-RA treatment (12, 13). Recently, it was shown that keratin K18 gene transcription in F9 teratocarcinoma cells is mediated through a c-fos regulatory response element (13), and that c-fos and c-jun are induced in F9 cells by RA (14). This suggests a possible basis for the delayed kinetics.

Little is known about the retinoid-mediated inhibition of keratin expression in epidermal cells. A fundamental prerequisite to understanding retinoid-mediated regulation of epidermal differentiation is a knowledge of the mechanisms underlying negative regulation of keratin expression in epidermal keratinocytes. To address this question, we examined the effects of RA treatment on protein expression, mRNA synthesis, and gene transcription in normal and squamous cell carcinoma cell line 13 (SCC-13) keratinocytes. Our results reveal some surprising differences between the RA-mediated changes in keratin gene expression in simple epithelial cells and those in epidermal cells.

EXPERIMENTAL PROCEDURES

Cell Culture. Primary human epidermal cells derived from newborn foreskins and the epidermal squamous cell carcinoma cell line SCC-13 (15) were cultured by the fibroblast feeder method (3), but omitting the triiodothyronine supplementation. A feeder-independent clone of SCC-13 cells was used for transfections and for the time course studies.

Protein, RNA, and Transcripts: Isolation and Analysis. Isolation and analysis of total proteins, [^{35}S]methionine-radiolabeled proteins, and total cellular RNA were performed as described (12). Immunoblot analyses of protein extracts were performed by using monospecific polyclonal antisera to K5 (E.F. and R. Lersch, unpublished data), K14, K6, and K16 (16), and a polyclonal rabbit antiserum to chicken actin (Biomedical Technologies, Stoughton, MA). ^{125}I-radiolabeled *Staphylococcus aureus* protein A (Amersham) was used to detect specifically bound IgG molecules. For Northern blot analysis of actin and keratin mRNAs, blots were hybridized with denatured ^{32}P-radiolabeled cDNA probes (3×10^5 cpm/ml), followed by washing three times for 30 min at 65°C in 0.1× SSC/0.1% SDS, pH 7.0 (1× SSC = 0.15 M NaCl/0.015 M sodium citrate). For analysis of RA receptor (RAR) mRNAs, blots were hybridized with ^{32}P-labeled cDNA probes (3×10^6 cpm/ml) and washed two times for 30 min at room temperature and two times for 30 min at 50°C in 0.1× SSC/0.1% SDS. Blots were then exposed to x-ray film. Nuclei isolation, transcript labeling, and analyses were carried out as described (12).

Plasmids. K5, K14, K6, K16, β-actin, and α-tubulin cDNA plasmids have been described as pK5-3′, pK14-3′, pK6-3′, pK16-3′, pAct1, and pTub1 (12). The insert sizes are as follows: K5, 189 base pairs (bp); K14, 282 bp; K6, 220 bp; K16, 350 bp; actin, 819 bp; tubulin, 1443 bp. cDNA se-

Abbreviations: RA, retinoic acid; CAT, chloramphenicol acetyltransferase; RAR, RA receptor.

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quences complementary to the 3′ ends of human K1 and K10 mRNAs were obtained by PCR with oligonucleotide primers designed from their published sequences (17, 18). The amplified cDNAs (K1, 473 bp; K10, 391 bp) were purified and inserted into the *Hin*cII or *Sma* I sites, respectively, of the plasmid pGem3Z (Promega). Identity of the clones was confirmed by sequencing (19). pK14CAT(−2300) has been described (20). pK5CAT(−6000) contains 6000 bp of sequences 5′ from the transcription initiation site of the human K5 gene subcloned into the plasmid pSV0CAT.PL1, a chloramphenicol acetyltransferase (CAT) reporter plasmid (E.F. and R. Lersch, unpublished data; see ref. 20). pACT-CAT, previously described as pHbAPr-1-CAT (21), contains 4300 bp 5′ upstream from the human β-actin gene linked to the CAT gene. pBLCAT-RARE contains three copies of a thyroid hormone/RA responsive element (22) inserted in front of the thymidine kinase promoter-driven CAT plasmid pBLCAT2 (23). Plasmids containing cDNA sequences of the RARs were gifts of P. Chambon (24).

Transfections and CAT Assays. Transfections and CAT assays were performed (20) with 6 pmol of CAT test plasmid, 3.5 μg of β-galactosidase control plasmid (pCH110), and carrier DNA to 45 μg (pKS; Stratagene). Cells were maintained for 48 hr in medium, with or without the addition of 1 μM RA, before harvesting.

RESULTS

RA Treatment Results in Decreased Keratin Protein Expression in Keratinocytes. Human epidermal keratinocytes grow optimally when cultured in medium containing ≈1.4 mM calcium, a condition that permits stratification and some features of terminal differentiation (25). Under these conditions, both epidermal and SCC-13 cells predominantly express K5, K6, K14, K16, and K17. To investigate the effects of RA on keratin expression relative to total protein expression, RA (1 μM) was added to cell cultures 0, 6, 24, 48, 72, and 96 hr before isolation of total proteins. Total proteins were resolved by SDS/PAGE and the proteins were visualized by staining with Coomassie blue (Fig. 1*A*). As judged from this gel, keratin proteins accounted for a much higher percentage of total proteins in normal keratinocytes than in SCC-13 cells. Moreover, keratin expression in SCC-13 cells was dramatically reduced by RA treatment.

Changes in protein synthesis were examined by radiolabeling cellular proteins by addition of 50 μCi of [^{35}S]methionine per ml of culture medium for 4 hr prior to isolation of protein. Films of the fluorographed gels are shown in Fig. 1*B*. Although SCC-13 cells were more sensitive to RA than epidermal keratinocytes, both cell types showed a decline in keratin synthesis relative to actin synthesis within 24 hr post-RA treatment. Thereafter, RA-mediated changes were not appreciable. The decline in keratin protein synthesis was reflected at the level of total keratin protein, as judged by immunoblot analysis (Fig. 1*C*). Thus, relative to actin levels, keratin protein levels began to decline within 24 hr post-RA treatment. Collectively, these data suggest that the differences in keratin expression between RA-treated epidermal and SCC-13 cells should be reflected at the levels of their mRNAs.

Changes in Keratin mRNA Levels Occur in Response to RA Treatment. Total cellular RNAs were isolated from RA-treated and untreated cells. Northern blot analysis was conducted with radiolabeled probes specific for β-actin and for the 3′ ends of K5, K14, K6, and K16 mRNAs (Fig. 2). Since overall keratin mRNA levels were greater in epidermal than in SCC-13 cells, longer exposures of the blots of SCC-13 mRNAs were required to visualize the changes. When either cell type was treated with RA, the levels of K5 [2.1 kilobases (kb)], K6 (2.1 kb), K14 (1.6 kb), and K16 (1.6 kb) mRNAs decreased relative to actin (1.9 kb) mRNA. As expected from

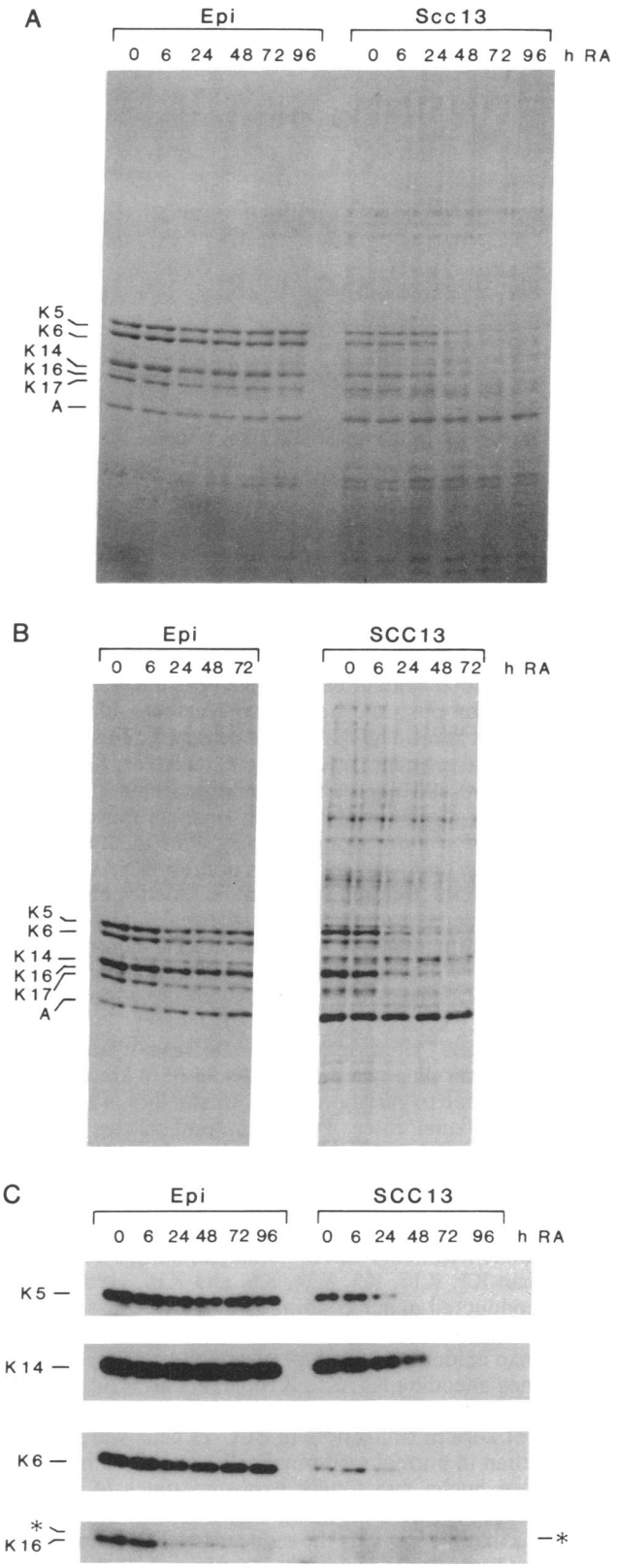

FIG. 1. Effects of RA on keratin protein and protein synthesis. Human epidermal cells (Epi) and SCC-13 keratinocytes were cultured in medium. RA was added for the times indicated. Four hours before harvesting, [^{35}S]methionine was added to the medium. Total protein samples (7.5 μg) were resolved by SDS/PAGE. (*A*) Coomassie blue staining of the gel. (*B*) Autoradiogram of the fluorographed gel shown in *A*. (*C*) Immunoblot analysis using antisera specific for either K5, K14, K6, or K16. All SCC-13 lanes contain 7.5 μg of protein. Sample sizes of epidermal cell protein are as follows: K5 and K14 blots, 1.9 μg; K6 blot, 3.8 μg; K16 blot, 7.5 μg.

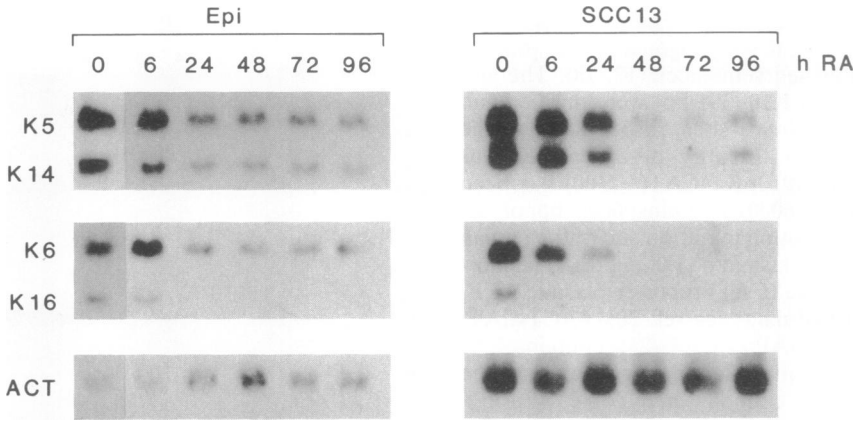

FIG. 2. Effects of RA on keratin mRNA expression. Cells were grown in medium containing 1.4 mM calcium. At the times indicated, RA was added before RNA isolation. Total RNAs (15 μg) were resolved by formaldehyde/agarose gel electrophoresis, transferred to nitrocellulose membranes, and probed with ^{32}P-radiolabeled cDNAs complementary to β-actin and the 3′ ends of K5, K14, K6, and K16 mRNAs. Epi, human epidermal cells. Exposure times: K5/K14 blots, Epi, 2 hr, SCC-13, 20 hr; K6/K16 blots, Epi, 15 hr, SCC-13, 96 hr; β-actin (ACT) blots, Epi, 6 hr, SCC-13, 22 hr.

the keratin synthesis patterns, the RA-mediated suppression of keratin mRNA expression was most pronounced for SCC-13 cells.

The kinetics of the RA-mediated response was examined by isolating total cellular RNAs from cells at t = 0, 6, 24, 48, 72, and 96 hr post-RA treatment. Northern blot analysis revealed that keratin mRNAs were largely diminished within 24 hr of RA treatment. Interestingly, however, K5 and K14 mRNAs were still appreciable in normal epidermal keratinocytes even after 96 hr of RA treatment. In contrast, SCC-13 cells showed a more dramatic reduction in these keratin mRNAs, with only very low levels of these RNAs remaining after 48 hr of RA treatment. The rapid kinetics observed for the RA-mediated reduction in epidermal and particularly SCC-13 keratin mRNAs were in striking contrast to the relatively slow kinetics of the RA-mediated enhancement of keratin mRNA expression previously observed for mesothelial and HeLa cells (4, 12).

Keratin Gene Transcription Rates Decrease upon RA Treatment. To determine whether the decrease in keratin mRNA levels were due to variation in mRNA stability or to variation in transcriptional rates, nuclear run-off assays were performed on nuclei isolated from epidermal and SCC-13 cells cultured in the presence or absence of RA. Nuclei were incubated in the presence of [^{32}P]UTP, and, after radiolabeling, transcripts were hybridized to filter-immobilized cDNA or genomic fragments corresponding to unique 3′ sequences of human K1, K10, K5, K14, K6, and K16. Hybridizations were conducted under conditions in which cross-hybridization with other transcripts was minimized (12).

Human epidermal cells had very high transcript levels for the genes encoding K5, K6, K10, K14, and K16, with lower transcript levels of the K1 gene (Fig. 3*A*, lane 1). The overall levels of keratin transcripts in SCC-13 cells were markedly lower than in normal epidermal cells, as judged by the need for more nuclei and longer exposure times to obtain the signals seen for SCC-13 cells (lanes 3 and 4). These findings indicate that the basis for the reduced levels of keratin protein in these tumor cells resides at least in part at the transcriptional level. For both epidermal cells and SCC-13 cells, RA markedly inhibited keratin gene transcription. This was most prominent for K1/K10 and K6/K16 transcripts, but it was also seen for K5/K14 transcripts. In contrast, neither tubulin nor actin transcription levels changed appreciably in response to RA treatment. Collectively, these data indicated that RA had a profound and specific inhibitory effect on keratin gene transcription in normal and in SCC-13 keratinocytes.

To determine whether the rapid changes in keratin mRNA expression were derived from rapid changes at the transcriptional level, we examined the kinetics of RA-mediated changes in keratin gene transcription. SCC-13 cells were treated with RA for 0, 6, 12, and 24 hr prior to nuclei isolation (Fig. 3*B*). As early as 6 hr post-RA treatment, transcriptional changes in keratin gene expression were detected (compare

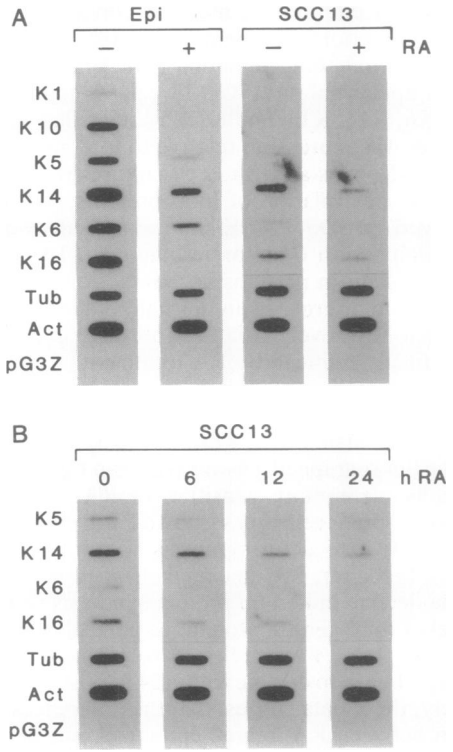

FIG. 3. Effects of RA on keratin gene transcription. (*A*) RA was added to culture medium 4 days before harvesting cells for nuclei isolation. For each assay, nuclei were harvested from 2.5 × 10^7 SCC-13 or from 1 × 10^7 normal epidermal (Epi) keratinocytes. Nuclei were incubated in the presence of [^{32}P]UTP and the radiolabeled RNA transcripts were isolated and hybridized to filter-immobilized cDNAs complementary to the mRNAs indicated. Blots were washed and exposed to x-ray film for either 3 days (Epi) or 14 days (SCC-13). (*B*) SCC-13 cells were grown in medium as described above, and RA was added for the times indicated before nuclei isolation. Nuclei were isolated and assayed as described above. pG3Z, pGEM3 plasmid control; Act, β-actin; Tub, α-tubulin.

lane 2 with lane 1). These changes were largely complete by 12–24 hr after RA treatment. Thus, the RA-mediated changes in keratin gene transcription were rapid.

Whether the greater sensitivity of SCC-13 cells to RA treatment is mediated solely at the transcriptional level, or whether some posttranscriptional regulation also contributes to this greater sensitivity of SCC-13 cells to RA, could not be ascertained with certainty from the nuclear run-off data. This was due to the fact that the keratin transcript signals in SCC-13 cells were extremely low and required long exposure times to be detected. The long exposure times enhanced the weaker keratin signals relative to the stronger actin and tubulin signals, and, thus, direct quantitative comparisons between epidermal and SCC-13 transcription were not possible.

Sequences in the 5′ Upstream Regions of the Human K14 and K5 Genes Mediate the Negative Transcriptional Regulation by Retinoids. The nuclear run-off data indicated that RA down-regulation of keratin expression is mediated at the transcriptional level. Previously, we showed that the plasmids pK14CAT(−2300), containing 2300 bp of human K14 gene upstream sequences and pK5CAT(−6000), containing 6000 bp of human K5 gene upstream sequences possess sufficient information to drive expression of a CAT reporter gene in cultured SCC-13 cells (ref. 20; R. Lersch and E.F., unpublished observations). To assess whether these constructs harbored information sufficient to confer RA-mediated transcriptional down-regulation to the CAT reporter gene, we transfected these plasmids into SCC-13 cells cultured in the absence or presence of RA. To control for variations between samples in transfection efficiencies, a simian virus 40 promoter/enhancer-driven β-galactosidase plasmid was cotransfected with each test plasmid. All CAT values were normalized accordingly.

SCC-13 cells treated with RA for 48 hr posttransfection exhibited levels of pK5CAT(−6000) expression, which were 15% those of untreated cells (Table 1). Similarly, RA treatment diminished expression of pK14CAT(−2300) to 40% the levels in untreated cells. In contrast, expression of a CAT construct, pACT-CAT, driven by a human β-actin promoter, was unaltered by RA treatment. Furthermore, RA caused elevated expression of a CAT construct, pBLCAT-RARE, containing elements known to positively respond to RA. These data extend the nuclear run-off experiments and suggest that cis-acting sequences involved in the RA-mediated down-regulation of keratin gene transcription are located upstream of the transcription initiation start sites of the human K14 and K5 genes.

RAR-γ and RAR-α, but Not RAR-β, mRNAs Are Expressed in Both Epidermal and SCC-13 Keratinocytes, and Their Levels Are Not Altered in Response to RA. RA exhibited rapid and marked changes in keratin gene transcription in keratinocytes. Since RARs are known to mediate a number of transcriptional events in various cell types (26, 27), it was of interest to examine RAR expression in these two cell types and to assess whether RA treatment might affect RAR expression in keratinocytes as it does for RAR-β in F9 teratocarcinoma cells (27). To address these questions, Northern blots of total RNAs (30 μg each) from RA-treated and untreated SCC-13 and epidermal cells were hybridized with radiolabeled probes specific for RAR-α, RAR-β, and RAR-γ mRNA. Our results revealed that RAR-γ mRNA (3.3 kb) was the predominant RAR mRNA expressed in keratinocytes (Fig. 4). Both cell types also expressed two RAR-α mRNAs of the expected sizes (3.8 and 2.8 kb; see ref. 24), but neither cell type expressed an mRNA detected with the RAR-β probe. No major differences were observed between RA-treated and untreated cultures, suggesting that keratinocytes differ from F9 teratocarcinoma cells in their response to RA. Relative to the levels of actin mRNAs, RAR-γ mRNA levels were higher in normal epidermal keratinocytes, while RAR-α was higher in SCC-13 cells. The extent to which these differences in RAR mRNA expression might account for the differences in RA sensitivity between epidermal and SCC-13 cells awaits further investigation.

Table 1. Effects of RA on expression of keratin–CAT constructs in transfected SCC-13 keratinocytes

Test plasmid	% CAT expression ± SEM		
	− RA	+ RA	+ DMSO
pK5CAT(−6000)	100	14.9 ± 8.3 (4)	ND
pK14CAT(−2300)	100	40.5 ± 12.0 (7)	98.8 ± 7.5 (6)
pACT-CAT	100	113.9 ± 19.4 (3)	ND
pBLCAT-RARE	100	659.6 ± 199.5 (2)	ND

CAT expression from cells not treated with RA represents 100% expression from each construct. The number of independent trials used to obtain each CAT value is shown in parentheses. ND, not determined; DMSO, dimethyl sulfoxide.

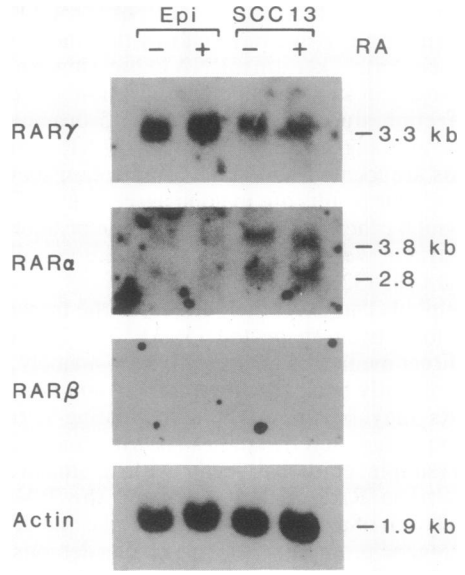

FIG. 4. Expression of RARs in epidermal and SCC-13 keratinocytes. Total RNAs (30 μg) isolated from RA-treated or untreated epidermal (Epi) or SCC-13 keratinocytes were resolved by formaldehyde/agarose gel electrophoresis, transferred to a nitrocellulose membrane, and probed with ^{32}P-radiolabeled cDNAs complementary to RAR-γ, RAR-α, RAR-β, and β-actin mRNAs. Blots were exposed to x-ray film for the following times: 3 days, RAR-α; 1 day, RAR-γ; 7 hr, β-actin; 7 days, RAR-β. Sizes of selected bands are as follows: RAR-γ, 3.3 kb; RAR-α, 3.8 and 2.8 kb; β-actin, 1.6 kb. Note that hybridization was not detected with the RAR-β probe.

DISCUSSION

Since the appearance of early reports describing the transformation of epidermis to a mucus-secreting epithelium upon treatment of organ cultures with an excess of vitamin A (2), retinoids have been suspected mediators of epidermal differentiation. Cultured epidermal cells respond to retinoids by suppressing their differentiation-specific morphology (3, 5, 28–30) and by decreasing expression of the terminal differentiation-associated keratins K1 and K10 (3, 11, 31) and the abnormal differentiation-associated keratins K6 and K16 (5, 9). Several reports have indicated that overall epidermal keratin protein levels may be lower in retinoid-treated cells (9, 10). Our results confirm this notion and imply that retinoids may influence keratin expression in basal as well as differentiating cells.

The RA-mediated effects that we observed at the level of keratin protein expression were also exhibited at the mRNA level, consistent with and extending previous studies show-

ing a RA-mediated reduction in K1 mRNA (3) and K6 mRNA (5). Most importantly, we have shown that the RA-induced changes in epidermal keratin mRNA expression are primarily due to changes in keratin gene transcription. Furthermore, our transfection studies with K14 and K5 promoter-driven CAT constructs suggest that sequences mediating the effects of retinoids are located within the 5′ upstream sequences of these genes. Our results are in good agreement with a recent study in which other epithelial keratin gene promoter-driven CAT constructs were transfected into cultured corneal and esophageal cells (32).

That retinoids can exert their effects at the transcriptional level has long been suspected (3, 33, 34). However, the putative direct mediators for this process—namely, RARs—have only recently been identified (ref. 24 and refs. therein). That RARs may be important in regulating keratin gene expression is suggested by studies showing that transfection of HeLa (simple epithelial) cells with a simian virus 40 promoter-driven RAR cDNA enhanced the RA-mediated down-regulation of a cotransfected CAT plasmid driven by a K14 pseudogene promoter (32). Our results demonstrate that RAR-α and RAR-γ mRNAs are expressed by both normal and SCC-13 epidermal cells and are thus likely candidates for mediating the RA response in these keratinocytes. Further studies will be necessary to ascertain whether the differences we have noted in RAR-γ and RAR-α mRNA levels in the two cell types account for their differences in RA sensitivity, or whether there might be additional factors involved in this process.

From the relatively fast kinetics of the RA-mediated effects on epidermal keratin gene expression, it is tempting to speculate that the response may be controlled by a direct interaction between a RAR–RA complex and sequences 5′ upstream of K5 and K14 genes. However, a fast response to a steroid hormone-like factor may not necessarily imply a direct receptor-mediated response, as indicated by the discovery of a protein synthesis-independent, negative regulatory mechanism involving the interaction between the glucocorticoid receptor and AP1 transcription factors (35–37). Conversely, a slow response to RA does not necessarily imply a non-RAR-mediated response, as evidenced by the laminin B1 gene, which shows relatively slow kinetics and yet is mediated by an apparent RAR response element (26). In this case, the explanation may reside in the fact that the RAR-β gene is itself inducible by RA and may be involved in triggering a number of relatively late RA-mediated responses (27, 38). Thus, retinoid-mediated control of gene expression is multifaceted and more complex than other steroid hormone-like responses.

Although we have not yet distinguished between direct and indirect mechanisms, we have shown that RAR-β is not appreciably induced by RA treatment of human epidermal cells cultured under the conditions described here. In addition, we uncovered kinetic differences between RA-mediated enhancement of simple epithelial keratin gene transcription (12) and RA-mediated suppression of epidermal keratin gene transcription. Collectively, these findings suggest that there may be tissue-specific differences in the mechanisms underlying the action of RA on keratin gene transcription. As further studies are conducted, and as the elements involved in RA regulation of various keratin genes are elucidated and characterized, the molecular basis for these differences should become more apparent.

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