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Differences in keratin synthesis between normal epithelial cells and squamous cell carcinomas are mediated by vitamin A

(gene regulation/mRNA synthesis/immunofluorescence/cultured cells/cellular retinoid binding proteins)

KWAN HEE KIM, FAINA SCHWARTZ, AND ELAINE FUCHS*

Department of Biochemistry, The University of Chicago, 920 East 58th Street, Chicago, IL 60637

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ABSTRACT **A number of recent studies have indicated that the expression of keratins is altered upon malignant transformation of human epithelial cells. We have shown that the altered expression of 67-kDa and 40-kDa keratins in established squamous cell carcinoma lines from tongue and epidermis stems largely from a difference in their sensitivity to vitamin A apparently acquired during tumorigenesis. When the vitamin A concentration in the medium is raised, the 40-kDa keratin is produced at increased levels. Conversely, when the amount of vitamin is reduced, the 67-kDa keratin is synthesized and the cells undergo stratification and terminal differentiation. However, even when vitamin A is quantitatively removed from the medium, the maximal degree of differentiation attained by each squamous cell carcinoma cell as judged by the synthesis of 67-kDa keratin was still less than that of the normal keratinocytes. These findings suggest that the altered patterns of keratins observed for some tissues upon malignant transformation arise from a complex mixture of intracellular changes in the differentiative pathway in addition to changes in the responsiveness of cells to extracellular regulators of keratin gene expression.**

Most vertebrate epithelial cells are dependent on vitamin A for their proper cellular differentiation and proliferation (for reviews, see refs. 1 and 2). When human keratinocytes derived from epidermis and other stratified squamous epithelia are cultured in medium supplemented with vitamin A-depleted serum, they undergo stratification and extensive terminal differentiation (3). Biochemically, major changes take place in the synthesis of keratins, the proteins that comprise abundant 8-nm cytoplasmic filaments in these cells. Removal of vitamin A from the culture medium leads to the synthesis of a 67-kDa keratin, one of the first *in vivo* indications that a keratinocyte has undergone a commitment to terminally differentiate (4–6). Simultaneously, there is a much reduced synthesis of the minor 40-kDa and 52-kDa keratins that are typically prominent in nonkeratinizing secretory epithelia—e.g., mesothelia, conjunctiva, trachea, and stomach (7, 8). The other four epidermal keratins of size 58, 56, 50, and 46 kDa show no change in synthesis in response to the vitamin. We have shown that vitamin A determines the nature of keratins synthesized by regulating the levels of their corresponding mRNAs (3).

The cell lines established from human squamous cell carcinomas (SCC) of epidermis and oral epithelium seem to exhibit a marked decrease in their commitment to terminally differentiate as manifested not only by a reduction in stratification, one of the earliest stages in the differentiative process, but also in the formation of cornified envelopes, one of the last steps in the pathway (9). These transformed cells produce almost no 67-kDa keratin but have unusually high levels of the 40-kDa keratin (10). Since it has been demonstrated that the synthesis of these two keratins is regulated by vitamin A, we wondered (*i*) whether the abnormal expression of the 40-kDa keratin in the carcinoma lines may be due to a change in the responsiveness of these cells to vitamin A and (*ii*) whether the cells can be induced to dfferentiate and stratify and to synthesize the 67-kDa keratin by reducing the level of vitamin A in the culture medium. These questions are particularly important to our understanding of the molecular mechanisms underlying changes in keratin gene expression upon malignant transformation of human epithelia (10–12). In the present study, we investigated the effects of vitamin A on keratin synthesis in two cultured human SCC lines, SCC-15 and SCC-12, and probed the basis for their altered expression of keratins.

MATERIALS AND METHODS

Cell Cultures. Human epidermal cell strains were derived from the foreskin of newborns. The SCC-15 and SCC-12 cells were derived from human SCC of tongue and skin, respectively, and have been described elsewhere (9). Cells were grown essentially according to the procedure of Rheinwald and Green (13), but under defined concentrations of retinyl acetate (3).

Antibodies. Antibodies against total cultured epidermal keratins (46–58 kDa) and against gel-purified keratins of 63 and 40 kDa were raised in rabbits according to the procedure outlined previously (4). Anti-63-kDa stratum corneum keratin antisera showed crossreactivity with the 67-kDa and 56-kDa keratins of cultured human epidermal cells. Anti-40-kDa keratin antisera showed strong crossreactivity with the 40-kDa keratin and weaker reactivity with all other epidermal keratins.

Immunofluorescence. Cells were fixed with 10% formalin in phosphate-buffered saline for 30 min at room temperature and then treated with methanol (−20°C) for 5 min. The cells were incubated at 37°C for 30 min with specific rabbit antiserum against human keratins (1:50 dilution), followed by 20 min with fluorescein-conjugated goat anti-rabbit IgG (Miles, 1:20 dilution).

Measurement of Cellular Retinoic Acid Binding Protein (CRABP). Cytosols from nine confluent 100-mm dishes of normal, SCC-12, and SCC-15 cells were labeled for 24 hr at 4°C with 50 nM [^{3}H]retinoic acid either alone or in the presence of a 25× excess of unlabeled retinoic acid. Bound and unbound retinoic acid were separated by the dextran-coated charcoal procedure, and the retinoic acid binding protein complexes were quantitated by sucrose gradient centrifugation (14). The amount of complex was determined by measuring the radioactivity in the peak at 2 S, its known sedimentation coefficient (15). Protein concentration in the cytosol was estimated by absorbance at 280 nm and by gel electrophoresis.

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Abbreviations: SCC, squamous cell carcinoma(s); CRABP, cellular retinoic acid binding protein.

*To whom reprint requests should be addressed.

Isolation and *in Vitro* Translation of Poly(A)$^+$ RNA. RNA was isolated by using the guanidine hydrochloride procedure outlined previously (16). mRNA was translated *in vitro* by using a rabbit reticulocyte cell-free system as described (16).

RESULTS

Altered Keratin Synthesis in SCC Cells. When grown to confluence in medium supplemented with 20% fetal calf serum, some established epithelial lines from SCC of epidermis (SCC-12; Fig. 1*a*, lane 4) and tongue (SCC-15, lane 2) produced a 40-kDa keratin in addition to the five keratins (46, 50, 52, 56, and 58 kDa) that are characteristic of keratinocytes cultured from normal tongue (lane 1) and epidermis (lane 3) (10). In contrast, the normal keratinocytes cultured from either tongue or epidermis did not synthesize any 40-kDa keratin, but did produce low levels of a 67-kDa keratin typical of terminally differentiating keratinocytes. Although no major tissue-specific differences in keratin biosynthesis were observed, it was noted that occasionally, normal tongue epithelial cells produced lower levels of 52-kDa keratin than epidermal cells and that only SCC-12 cells produced a 54-kDa protein. Since these minor differences were not routinely observed, they were not further investigated.

Effect of Vitamin A Deficiency on Keratin Synthesis in SCC Cells. Vitamin A can be largely removed (<1 nM) from fetal calf serum by extraction with polar solvents (3, 17). The concentration of vitamin A in medium prepared from untreated serum was determined by high-pressure liquid chromatography to be 0.52 μM (18). When normal stratified squamous epithelial cells cultured from tongue or epidermis were grown in vitamin A-depleted medium, the cells stratified and produced increased levels of the 67-kDa keratin (Fig. 1*b*, lanes 2–4). Surprisingly, when SCC cells from these tissues were grown to confluence and maintained in medium supplemented with lipid-extracted serum, they also began to differentiate, stratify, and produce the 67-kDa keratin (Fig. 1*c*, lanes 2–4). For all cell types, the extent of differentiation and 67-kDa keratin synthesis reached a maximum at 2 wk after confluence. In the SCC cells, the appearance of the 67-kDa keratin was somewhat delayed and was less than that of the normal cells, even though both cell types had been cultured in the same medium. The synthesis of the 40-kDa keratin showed behavior opposite to that of the 67-kDa keratin (Fig. 1*c*, lanes 2–4). After several weeks at confluence in vitamin A-depleted medium, no 40-kDa keratin synthesis could be detected (lanes 3 and 4), even though this keratin had been a major constituent of SCC cells grown in medium containing untreated serum (lanes 5–7). Similarly, another keratin of 52 kDa also showed decreased synthesis in the presence of delipidized medium. Collectively, these results indicate that the vitamin A-mediated control mechanism operating on the biosynthesis of the 40-, 52-, and 67-kDa keratins is acting on both the normal and the SCC cells. Moreover, the expression of the 40-kDa keratin in the SCC lines is transient and is clearly dependent on the amount of vitamin A in the culture medium.

Dose-Dependent Response of SCC Cells to Retinyl Acetate. Similar to normal keratinocytes, tumor cells showed a dose-dependent decrease in the synthesis of 67-kDa keratin and a concomitant increase in the levels of 40-kDa and 52-kDa keratins when vitamin A in the form of retinyl acetate was added to the delipidized medium at concentrations ranging from 35 nM to 1.5 μM (Fig. 2). However, for each concentration of retinyl acetate, the relative amounts of 40-kDa and 67-kDa keratins synthesized by the SCC cells were usually equivalent to that produced by the normal keratinocytes grown in about 10× higher concentrations of the vitamin. This apparent increased sensitivity of SCC cells to vitamin A was observed for both SCC-12 cells of epidermal origin and SCC-15 cells cultured from a SCC of the tongue.

The relative differences in the amounts of 67-kDa and 40-kDa keratins produced by the normal and tumor cells under identical growth conditions can be best visualized by indirect immunolocalization of these proteins following their electrophoretic separation (Fig. 3). In an immunoblot analysis, the presence of 67-kDa keratin in both normal and tumor cells grown in vitamin A-deficient medium could easily be detected (lanes 2 and 4). The level of 67-kDa keratin produced by the normal cells was clearly higher than that in the tumor cells. For both cell types, the addition of retinyl acetate to the medium resulted in the disappearance of this keratin (Fig. 3*a*, lanes 3 and 5). In contrast, the 40-kDa keratin was

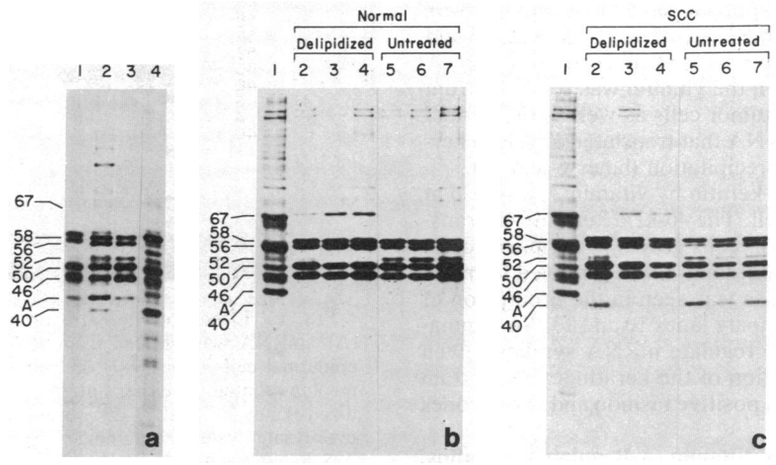

FIG. 1. Keratins of cultured human epithelial cells and SCC lines and their time-dependent response to vitamin A. (*a*) Human tissues and cells from different sources were labeled *in vitro* with [^{35}S]methionine and the keratins were extracted. Proteins were resolved by electrophoresis through 8.5% polyacrylamide gels, which were subsequently fluorographed and exposed to film. Keratins were extracted from normal cultured tongue epithelial cells (lane 1); SCC-15, a tongue SCC line (lane 2); normal cultured epidermal cells (lane 3); and SCC-12, an epidermal SCC line (lane 4). (*b* and *c*) The data shown here are for human epidermal cells (*b*) and SCC-15 cells (*c*) although the results obtained for cultured human tongue epithelial cells were found to be indistinguishable from *b* and those for SCC-12 cells were identical to *c*. Cells were cultured in medium containing either untreated or delipidized serum. When the cells were 75% confluent and at intervals of 1 wk thereafter, cells were labeled *in vivo* with [^{35}S]methionine and the keratins were extracted. Lanes 1, keratin markers from human epidermal tissue showing the 67-kDa keratin; lanes 2, keratins extracted from cells cultured in delipidized medium until 75% confluent; lanes 3, 1 wk after confluence; lanes 4, 2 wk after confluence; lanes 5, keratins extracted from cells cultured in untreated medium until 75% confluent; lanes 6, 1 wk after confluence; and lanes 7, 2 wk after confluence. For *a–c*, numbers at the left represent molecular size in kDa. A, actin.

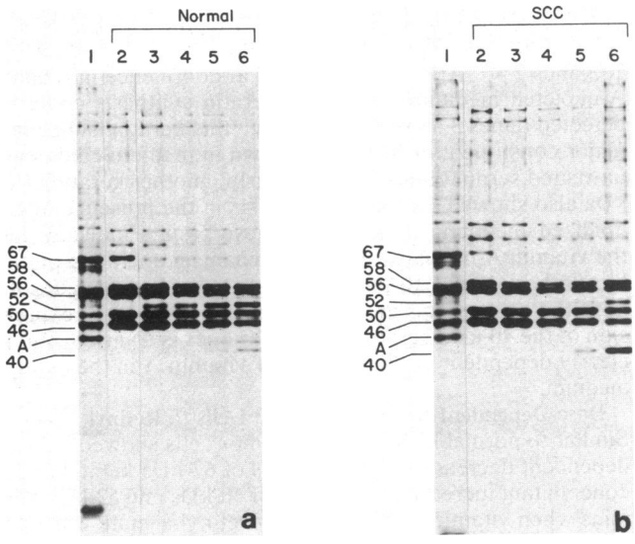

FIG. 2. Vitamin A dose-dependent relation between the 67-kDa and 40-kDa keratins. Results shown are for human epidermal (*a*) and SCC-15 (*b*) cells, although human tongue cells gave similar results to *a* and SCC-12 cells showed behavior indistinguishable from *b*. Cells were grown in medium containing delipidized serum and a defined concentration of retinyl acetate. After cells were maintained in their appropriate medium for 2 wk after confluence, they were labeled with [^{35}S]methionine and the keratins were extracted. Proteins were separated on an 8.5% polyacrylamide gel, which was then fluorographed and autoradiographed. Keratins were extracted from epidermis (lanes 1) and from cells cultured in delipidized medium containing retinyl acetate at final concentrations of 0 (lanes 2), 35 nM (lanes 3), 0.15 μM (lanes 4), 0.35 μM (lanes 5), and 1.5 μM (lanes 6). Numbers represent molecular size in kDa. A, actin.

produced at a very low level in normal cells cultured in medium containing 1.5 μM retinyl acetate (Fig. 3*b*, lane 3), but it was a predominant keratin in the tumor cells cultured in the same medium (lane 5). Keratins of 46, 50, 56, and 58 kDa showed no significant change (Fig. 3*c*).

Effect of Retinyl Acetate on Keratin mRNA Synthesis in SCC Cells. As judged by immunoprecipitation with a general antikeratin antiserum, no appreciable 67-kDa keratin was translated *in vitro* from the mRNA isolated from SCC-15 and normal epidermal cells grown in the presence of vitamin A (Fig. 4, lanes 3 and 9). When the vitamin was removed from the medium, however, the tumor cells as well as the normal keratinocytes produced mRNA that translated a 67-kDa keratin identified by immunoprecipitation (lanes 6 and 12).

Regulation of the 40-kDa keratin by vitamin A was also at the level of mRNA in the cell. The 40-kDa keratin was translated from mRNA of SCC-15 cells grown in the presence of untreated serum, but when vitamin A was removed from the medium, a dramatic decrease was seen in the production of 40-kDa keratin mRNA (compare lanes 10 and 13). In summary, vitamin A continued to regulate mRNA synthesis even after malignant transformation of the keratinocytes: certain mRNAs were regulated in a positive fashion and certain ones in a negative fashion.

Relationship Among the Vitamin A-Regulated Keratins. The keratins are expressed from two families of genes (19). These two classes of keratin sequences, type I and type II, have been shown to be only distantly related but are thought to form the backbone of the protofibril of the 8-nm keratin filament (20, 21). The 40-kDa and 52-kDa keratins have been classified as type I keratins according to the ability of their mRNAs to hybridize with a cloned cDNA for the type I human epidermal 50-kDa keratin (22). Other small and acidic keratins have also been identified as type I keratins, whereas the larger and more basic keratins usually have similarities

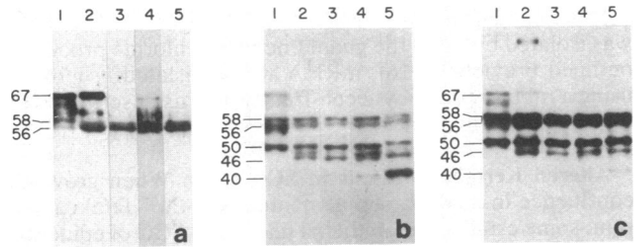

FIG. 3. Immunolocalization of keratins in normal and tumor cells grown in medium containing varying levels of retinyl acetate. Protein extracts from normal epidermal cells and SCC-15 cells grown in medium containing delipidized serum supplemented with either 0 or 1.5 μM retinyl acetate were resolved by polyacrylamide gel electrophoresis in triplicate. Proteins from each gel were electrophoretically transferred to nitrocellulose paper and identified by immunoreactivity with specific antisera against 63-kDa stratum corneum keratin (*a*), 40-kDa keratin (*b*), and 46- to 58-kDa epidermal keratins (*c*). Immunoreaction was visualized by indirect localization with ^{125}I-labeled *Staphylococcus aureus* protein A. Proteins were from epidermis (lanes 1), epidermal cells cultured in medium containing delipidized serum (lanes 2), epidermal cells cultured in medium containing delipidized serum and 1.5 μM retinyl acetate (lanes 3), and SCC-15 cells cultured as in lanes 2 and 3 (lanes 4 and 5, respectively).

with the type II class (22). We show in Fig. 5 that a large (≈3.8 kilobases) mRNA synthesized by cells grown in vitamin A-depleted medium (lane 1) but not by cells grown in normal medium (lane 2) hybridized only with the type II keratin cDNA probe. Although this mRNA has not yet been conclusively identified, it is certainly a likely candidate for

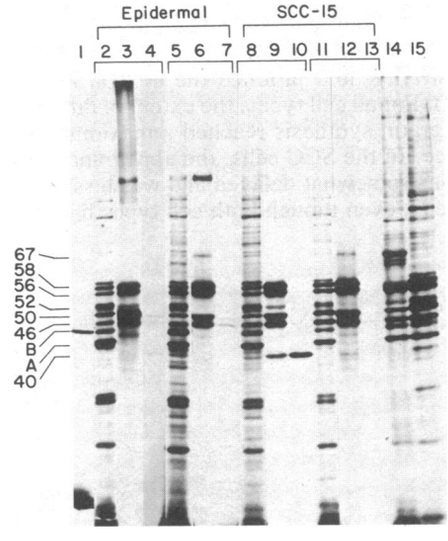

FIG. 4. Effects of vitamin A on keratin mRNA synthesis. Poly-(A)$^+$ mRNA was isolated from 75% confluent cultures of human epidermal cells (lanes 2–7) or SCC-15 cells (lanes 8–13) grown in medium containing either untreated (lanes 2–4 and 8–10) or delipidized (lanes 5–7 and 11–13) serum. After translation in a reticulocyte-lysate system containing [^{35}S]methionine, the products (lanes 2, 5, 8, and 11) were precipitated with antisera specific for either the major keratins of epidermal cells (lanes 3, 6, 9, and 12) or the 40-kDa keratin (lanes 4, 7, 10, and 13). Proteins were analyzed by electrophoresis and autoradiographed. Lane 1, B, an mRNA-independent artifact of the reticulocyte-lysate system; lane 14, keratins from human back epidermis showing the 67-kDa keratin; lane 15, keratins from SCC-15 cells showing the 40-kDa keratin. Numbers at the left represent molecular sizes in kDa. A, actin. Note that a large molecular size band (>100 kDa) (lanes 3 and 6) is probably an artifact of the immunoprecipitation since (*i*) this band has not been seen previously and (*ii*) the possibility of a large precursor keratin mRNA has already been ruled out (16).

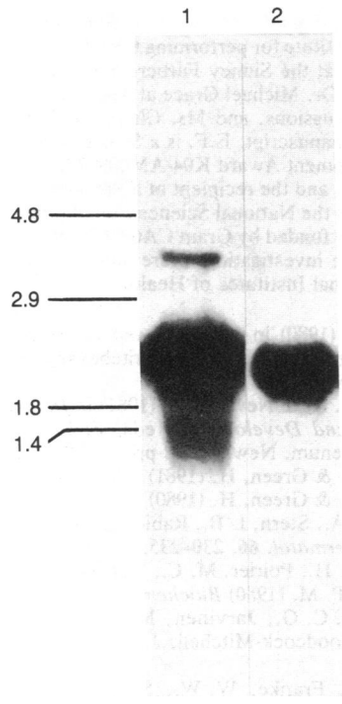

FIG. 5. Preliminary identification of 67-kDa keratin mRNA by hybridization with a type II keratin cDNA probe. Aliquots (1 μg) of poly(A)$^+$ mRNA from epidermal cells cultured in delipidized medium (lane 1) and in untreated medium (lane 2) were loaded in the sample wells of a 0.8% agarose/formaldehyde RNA gel (23). Eukaryotic rRNA (18 S and 28 S) and *Escherichia coli* rRNA (16 S and 23 S) were used as markers and their molecular sizes are indicated at the left in kilobases. Following electrophoresis, RNA was transferred to nitrocellulose paper (24) and the filter was hybridized under stringent conditions (0.75 M NaCl/50% formamide, 41°C) with ^{32}P-labeled probe copied (by using reverse transcriptase) from a cloned type II keratin (56 kDa) cDNA (19). After washing, the filter was exposed to x-ray film with an intensifying screen.

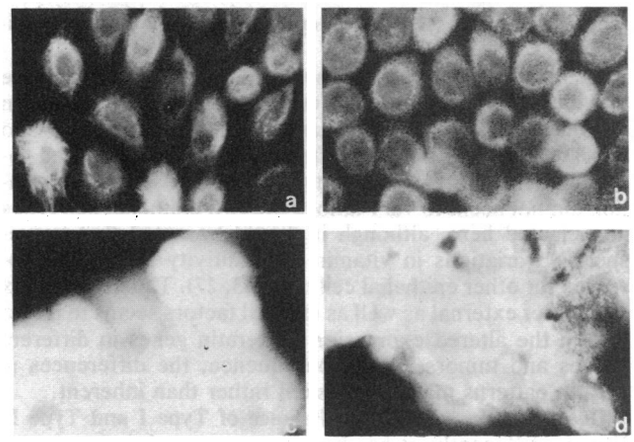

FIG. 6. Immunofluorescence detection of the 67-kDa keratin in differentiating epidermal cells. Epidermal cells (*a* and *c*) or SCC-15 cells (*b* and *d*) were grown in the presence (*a* and *b*) or absence (*c* and *d*) of vitamin A. Keratin filaments were stained *in situ* by fluorescein-conjugated antiserum made against the 63-kDa keratin of stratum corneum (4). Stained cultures were viewed in a Zeiss epifluorescence microscope with a 100× oil immersion objective. *a–d* were taken with a 15-sec exposure and then processed in exactly the same manner. Preimmune sera showed no detectable staining under these conditions.

the 67-kDa keratin mRNA, and it seems that this large keratin belongs to the type II class of sequences.

Indirect Immunofluorescent Localization of the 67-kDa Keratin in Terminally Differentiating Cells. When cells were grown in vitamin A-depleted medium, the level of 67-kDa keratin synthesized by SCC cells was always slightly lower than that of the normal keratinocytes. This variation may arise from a difference in the fraction of cells undergoing terminal differentiation. Alternatively, it may reflect a difference in the extent of differentiation attained by each cell within the population. To distinguish between these two possibilities, indirect immunofluorescence was used to measure the variability in 67-kDa keratin within the individual cells of the population (Fig. 6). When normal and SCC keratinocytes were grown for 2 wk after confluence in medium containing untreated serum, an anti-63-kDa stratum corneum keratin antiserum showed only limited staining of the keratin filaments (Fig. 6 *a* and *b*). Some of this staining was probably due to the weak crossreactivity of the antisera with the 56-kDa keratin. Since many of the normal cells (Fig. 6*a*) repeatedly stained slightly better than the tumor cells (Fig. 6*b*), the difference most likely reflects the low levels of 67-kDa keratin that appear in normal epidermal cells as they remain postconfluent in serum-containing medium.

When both cell types were grown in vitamin A-depleted medium, extensive terminal differentiation occurred and a marked increase in fluorescence was seen in >70% of the cells (Fig. 6 *c* and *d*). This increase in antibody-specific staining was most certainly due to newly synthesized 67-kDa keratin, which was also easily detected in both cell extracts (see also Fig. 1). However, the normal cells still stained brighter than the tumor cells, indicating that the entire population of differentiating SCC cells was producing less 67-kDa keratin than were the differentiating normal cells. Thus, as judged by the amount of 67-kDa keratin synthesis, SCC cells showed a reduction in the extent of terminal differentiation typically seen in normal keratinocytes.

Measurement of CRABP in Normal and SCC Cells. A number of recent reports have suggested that a cytoplasmic 14-kDa protein, CRABP, plays an important role in the mechanism of action of retinoids on epithelial differentiation (for a review, see ref. 25). To determine whether the effects of retinyl acetate on keratin biosynthesis might be mediated by CRABP, we measured the levels of this protein in normal and SCC cells. The levels (mean ± SEM) of CRABP in SCC-15 cells, SCC-12 cells, and normal cultured human epidermal keratinocytes were 6.8 ± 0.5, 3.5 ± 2.2, and 13.5 ± 1 pmol/mg of protein, respectively. These levels are all considered to be high, although the amount in the normal cells was consistently about 2- to 3-fold higher than that in the SCC lines. This trend was similar to that noted recently for esophageal tissue, in which the level of CRABP was higher in the adjacent normal mucosa than in the SCC (26).

DISCUSSION

Vitamin A Regulates Changes in Keratin Expression upon Malignant Transformation. SCC lines derived from human tumors of the epidermis and oral epithelium have been shown to contain an internal defect in terminal differentiation (9). Our results have shown that these cells have acquired an increased sensitivity to the differentiation inhibitor vitamin A and, thus, the temporal appearance of the block in the differentiative pathway occurs at a later stage than was initially realized. The early events in this pathway—i.e., stratification and subsequent synthesis of the 67-kDa keratin—can be triggered in SCC cells provided that the level of vitamin A is reduced in the culture medium. However, an internal block late in the differentiative pathway of the SCC cells must also exist, since the amount of 67-kDa keratin synthesis in the SCC cells is less than in the normal cells even

when vitamin A is virtually depleted from the culture medium.

In this study, we have presented a well-documented case in which altered keratin expression in tumor cells has been attributed in large part to a change in their responsiveness to an external modulator of differentiation. The acquired increase in vitamin A sensitivity upon malignant transformation did not seem to vary among the two epithelial cell types investigated here, although it should be noted that tissue-specific variations in vitamin A sensitivity have been observed for other epithelial cell types (3, 27). Thus, a complex mixture of external as well as internal factors seems to play a role in the altered expression of keratin genes in different tissues and tumors. As a consequence, the differences in keratin patterns may be transient rather than inherent.

Does Vitamin A Shift the Balance of Type I and Type II Keratins? Recently, it has been demonstrated that a combination of type I and type II keratins may be important and possibly essential for filament assembly (19, 28–32). Thus, it was intriguing to discover that the two major vitamin A-related keratin mRNAs appear to belong to two distinct sequence classes, suggesting that the ratio of type II to type I keratins might be increasing during terminal differentiation of the keratinocyte. However, an additional, albeit minor, type I keratin mRNA of size (2.5 kilobases) similar to that encoding the 56- to 58-kDa type II keratins (19) also appeared in the vitamin A-depleted cell mRNA (data not shown). This mRNA might encode the 56.5-kDa keratin previously described for differentiating human epidermal cells (4, 28). Thus, it is possible that the commitment of a cell to terminally differentiate, which is triggered by depletion of vitamin A, might result in a shift to the synthesis of larger keratins of both types without necessarily disrupting the intracellular ratio of the type II to type I keratins.

Since two major type II keratins (56 and 58 kDa) and two major type I keratins (46 and 50 kDa) are continuously synthesized by the cells regardless of vitamin A concentration, it is unlikely that any of the vitamin A-mediated changes described in this study would interfere with filament assembly. Nonetheless, the shift to the production of unusually large keratins might be responsible for the altered morphology of the keratin filament network inside the differentiating cell. These large keratins may also play a role in the cessation of DNA synthesis and mitosis during terminal differentiation.

Role of CRABP in the Action of Vitamin A. Recent experiments with embryonal carcinoma cell mutants lacking CRABP have shown that the action of vitamin A in these cells is mediated by this protein (33). Our results have demonstrated that the differences in the intracellular levels of CRABP do not parallel the relative differences in the sensitivity of the cells to the vitamin. This does not rule out the possibility that CRABP is the mediator of the response of keratinocytes to vitamin A; however, it does imply that the rate-limiting step of vitamin A action cannot be the formation of CRABP–retinoid complexes in these cells. It could be that the relative sensitivity to the vitamin is determined by the number of cell surface receptors for the plasma binding protein–vitamin A complex. Alternatively, one or more of the many cell surface changes known to take place upon malignant transformation may allow a greater fraction of extracellular retinyl acetate to enter the cell. Whatever this change might be, the precise sequence of biochemical events that are triggered by the presence or absence of vitamin A has not been disrupted by malignant transformation of the keratinocytes of human epidermis and oral epithelia.

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