The Use of Retinoic Acid To Probe the Relation Between Hyperproliferation-associated Keratins and Cell Proliferation in Normal and Malignant Epidermal Cells

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Abstract. When cells from normal human epidermis and from the human squamous cell carcinoma line SCC-13 were seeded on floating rafts of collagen and fibroblasts, they stratified and underwent terminal differentiation. Although the program of differentiation in SCC-13 cells was morphologically abnormal, the cultures resembled normal epidermal raft cultures by expressing the terminal differentiation-specific keratins, K1/K10, and by restricting their proliferative capacity to the basal-like cells of the population. In addition, the differentiating cells of both normal and SCC-13 raft cultures expressed keratins K6 and K16, which are not normally expressed in epidermis, but are synthesized suprabasally during wound-healing and in various epidermal diseases associated with hyperproliferation. While the behavior of normal and SCC-13 rafts was quite similar when they were cultured over normal medium, significant biochemical differences began to emerge when the cultures were exposed to retinoic acid. Most notably, while the SCC-13 cultures still stratified extensively, they showed a marked inhibition of both abnormal (K6/K16) and normal (K1/K10) differentiation-associated keratins, concomitantly with an overall disappearance of differentiated phenotype. Surprisingly, the reduction in K6/K16 in retinoid-treated SCC-13 cultures was not accompanied by a decrease in cell proliferation. Using immunohistochemistry combined with [^{3}H]thymidine labeling, we demonstrate that while the expression of K6 and K16 are often associated with hyperproliferation, these keratins are only produced in the nondividing, differentiating populations of proliferating cultures. Moreover, since their expression can be suppressed without a corresponding decrease in proliferation, the expression of these keratins cannot be essential to the nature of the hyperproliferative epidermal cell.

THE normal epidermis consists of 10–20 layers whose cells possess distinct structure and properties. Only cells in the basal layer proliferate (Weinstein and Van Scott, 1965). As cells leave this layer and begin to migrate towards the skin surface, they cease to divide and start to terminally differentiate. The cells enter the spinous layer and increase steadily in size as they continue to be metabolically active (Yardley and Goldstein, 1976). During this phase, much of the cell's translational machinery is devoted to making keratins, the major product of the differentiated epidermis (Matoltsy, 1975). These proteins assemble into 8-nm filaments that increase in number and aggregate into bundles in the cytoplasm of the differentiating cell (Brody, 1960). As cells reach the granular layer, they enter a destructive phase and begin to lose their organelles, leaving keratin filaments as virtually the sole cytoplasmic survivors of the differentiative process (Lavker and Matoltsy, 1970). Released from the skin surface, the dead squames are continually being replenished by differentiating cells migrating outward.

A number of changes in protein synthesis take place during terminal differentiation. The major change is the induction of unusually large keratins (Fuchs and Green, 1980), which occurs as a cell leaves the basal layer (Viac et al., 1980). While basal cells express keratins K5 (58 kD) and K14 (50 kD) (Nelson and Sun, 1983), suprabasal cells express keratins K1 (67 kD), K2 (65 kD), K10 (56.5 kD), and K11 (56 kD) (Fuchs and Green, 1980; Eichner et al., 1984). With the possible exception of K2 and K11, which may be proteolytic degradation products of K1 and K10, respectively (Fuchs and Green, 1980; Tyner and Fuchs, 1986), the shift to the expression of these large keratins is due to changes in specific keratin mRNAs (Fuchs and Green, 1980). The appearance of large keratin mRNAs is one of the earliest biochemical indications that a cell has undergone a commitment towards terminal differentiation.

In a number of epidermal diseases associated with hyperproliferation, the expression of these large keratins is downregulated and a new set of keratins, K6 (56 kD) and K16 (48 kD), is induced (Weiss et al., 1984; McGuire et al., 1984). Similar to the large keratins, K6 and K16 are also located in the suprabasal epidermal cells (Stoler et al., 1988). Coincidently, [^{3}H]thymidine labeling of hyperproliferative epider-

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mis such as psoriatic tissue has shown that the number of cells synthesizing DNA is greatly enhanced in the suprabasal layers (Van Scott and Ekel, 1963; Weinstein and Van Scott, 1965; Penneys et al., 1970; Leigh et al., 1985). However, even though a correlation between hyperproliferation and K6/K16 expression is well-documented, the extent to which the expression of these keratins plays a functional role in the process of epidermal cell proliferation remains to be determined.

The focus of the present study was to examine the relation between K6/K16 expression and epidermal proliferation. We used as model systems normal and malignant human epidermal cells cultured in vitro under conditions where they stratify and differentiate. Previously, we showed that when normal human epidermal cells or cells from the epidermal squamous cell carcinoma line SCC-13 are seeded on floating rafts of collagen and fibroblasts, they express both sets of suprabasal keratins in addition to the basal keratins (Kopan et al., 1987; Stoler et al., 1988). Hence, the biochemical processes which occur in vivo in response to epidermal proliferation also take place in vitro when this culture system is used.

We chose to examine both normal and SCC-13 cells, since the two cell types have different proliferative potentials (Wu and Rheinwald, 1981; Albers and Taichman, unpublished results). However, we also wanted a method which would enable us to manipulate both the process of differentiation and also the proliferative potential within an individual cell type. With respect to suppressing terminal differentiation, the task was relatively simple, since we knew that when retinoic acid is added to the medium underlying floating rafts of normal epidermal cultures, the expression of the terminal differentiation-specific keratins, K1 and K10, is suppressed (Kopan et al., 1987). While K6 and K16 are still synthesized in normal epidermal cultures, their expression also appeared to be reduced somewhat in the presence of retinoids (see two-dimensional gels in Kopan et al., 1987). Although we had not yet examined the effects of retinoic acid on raft cultures of SCC-13 cells, we had shown previously that when similar cell lines SCC-12 and SCC-15 (Wu and Rheinwald, 1981) were cultured on plastic in medium containing retinoic acid, they seemed to be even more sensitive to retinoids than normal keratinocytes (Kim et al., 1984). Hence, we anticipated that the SCC-13 raft cultures would be responsive to retinoic acid.

The question of whether retinoids inhibit or stimulate epidermal proliferation has been a matter of considerable controversy (Yuspa and Harris, 1974; Chopra and Flaxman, 1975; Hardy et al., 1975; Marcelo and Madison, 1984; Hashimoto et al., 1985; Connor, 1986; Redfern and Todd, 1988). However, preliminary studies examining relative rates at which normal human keratinocytes reach confluence when cultured on plastic in the presence of varying concentrations of retinoids have suggested a possible basis for the controversy: low levels of retinoids seemed to stimulate proliferation, whereas higher levels seemed to be inhibitory (Fuchs and Green, 1981). Thus, by using retinoids to alter the proliferation of normal and transformed human epidermal keratinocytes in vitro, we hoped to gain some insight as to the role of K6/K16 expression in hyperproliferative tissues.

Specifically, the questions that we addressed in this study are: (*a*) How does the level of DNA synthesis differ in normal epidermal raft cultures and SCC-13 raft cultures? (*b*) Which cells of the stratified population of epidermal and SCC-13 cultures are synthesizing DNA? (*c*) Do DNA-synthesizing cells of the raft cultures express K6/K16? (*d*) Does retinoic acid influence the expression of K6/K16 in the raft cultures? (*e*) Does retinoic acid influence the level of DNA synthesis in raft cultures? (*f*) Can K6/K16 expression and epidermal cell proliferation be uncoupled? (*g*) What are the mechanisms underlying the control of K6/K16 expression in culture?

Materials and Methods

Preparation of Floating Epidermal Colonies on Fibroblast/Collagen Lattices

The procedure for making and seeding collagen lattices was essentially as that described by Asselineau et al. (1985). Type I collagen was combined with culture medium at 4°C as described by the manufacturer (Seikagaku America, Inc., St. Petersburg, FL). Mouse 3T3 fibroblasts (Rheinwald and Green, 1975) were added to this solution at a concentration of 1.5×10^5/ml, and the mixture was pipetted into 35-mm culture dishes (2 ml/dish). To gel, lattices were placed in the incubator at 37°C. After 2–3 h, medium was added at 2 ml/dish, and lattices were maintained in the incubator until ready for use (usually 12–72 h after gellation). Cultures were fed with a 3:1 mixture of DME and Ham's F12 medium, supplemented with hydrocortisone (0.4 μg/ml) and 10% FCS (HyClone Laboratories, Logan, UT).

To prepare epidermal cultures, each dermal lattice was seeded with 3×10^5 human SCC-13 keratinocytes (Rheinwald and Beckett, 1981) or normal epidermal cells (derived from foreskin and kept as 3-d passage-cloned stocks under liquid nitrogen; Rheinwald and Green, 1975; Rheinwald, 1980). Cells were grown submerged in culture medium for 7 d. At this point, lattices were removed from the dishes and placed on stainless steel grids (Asselineau et al., 1985,1986; Kopan et al., 1987). Grids were floated on medium for 10 d before harvesting. Where indicated in the text, 1×10^{-7} to 1×10^{-6} M retinoic acid (Sigma Chemical Co., St. Louis, MO) was added to this medium at the time the cultures were raised to the air-liquid interface. Retinoic acid was stored at −70°C in the dark as 400× stocks in dimethyl sulfoxide, and diluted into medium at each feeding. Control plates with no retinoic acid still received a 1:400 dilution of dimethyl sulfoxide into the medium.

[^{3}H]Thymidine Labeling of Cell Cultures

Cells were cultured on rafts for 10 d before labeling. 2 h before fixation, submerged rafts (controls) or floating rafts (experiment) were labeled with 2 μCi/ml of [^{3}H]thymidine (89 Ci/mmol; Amersham Corp., Arlington Heights, IL) were added to the medium. After returning cultures to the incubator for 2 h, raft cultures were thoroughly washed in PBS, and then fixed in Carnoy's solution for 30 min. Fixed cultures were embedded in paraffin and sectioned (5 μm).

Autoradiography and Hematoxylin/Eosin Staining of Raft Cultures

Sections of fixed tissues were adhered to glass slides, and slides were dipped in xylene to deparaffinize the sections. After two changes of 100% xylene and one of 100% ethanol (10 min each), slides were air-dried. From this point until after developing, slides were kept in complete darkness. Slides were first dipped in NTB2 Kodak emulsion as described by the manufacturer. Dipped slides were air-dried for 90 min, and then stored at 4°C in a light-proof and radiation-proof lead bag for 3 wk before developing. The developing was done in 1/2× Dektol (Kodak) at 14°C for 4 min (the developer should be diluted with glass distilled water). Developing was stopped by dipping slides in 0.01 M glacial acetic acid and fixed in Kodak GBX fixer for 4 min at 14°C. Slides were washed in cold running tap water for 15 min before staining with filtered 100% hematoxylin (Sigma Chemical Co.). After ethanol dehydration, slides were stained with 0.05% eosin (in 70% ethanol, 0.5% glacial acetic acid), washed in 100% ethanol, immersed in xylene, and mounted under glass cover slips with Pro-Texx (Lerner Laboratories, Pittsburgh, PA).

Immunohistochemistry

Fixed, paraffin-embedded sections (5 μm) were hydrated before staining. Sections were treated for 30 min at room temperature with a solution of 2%

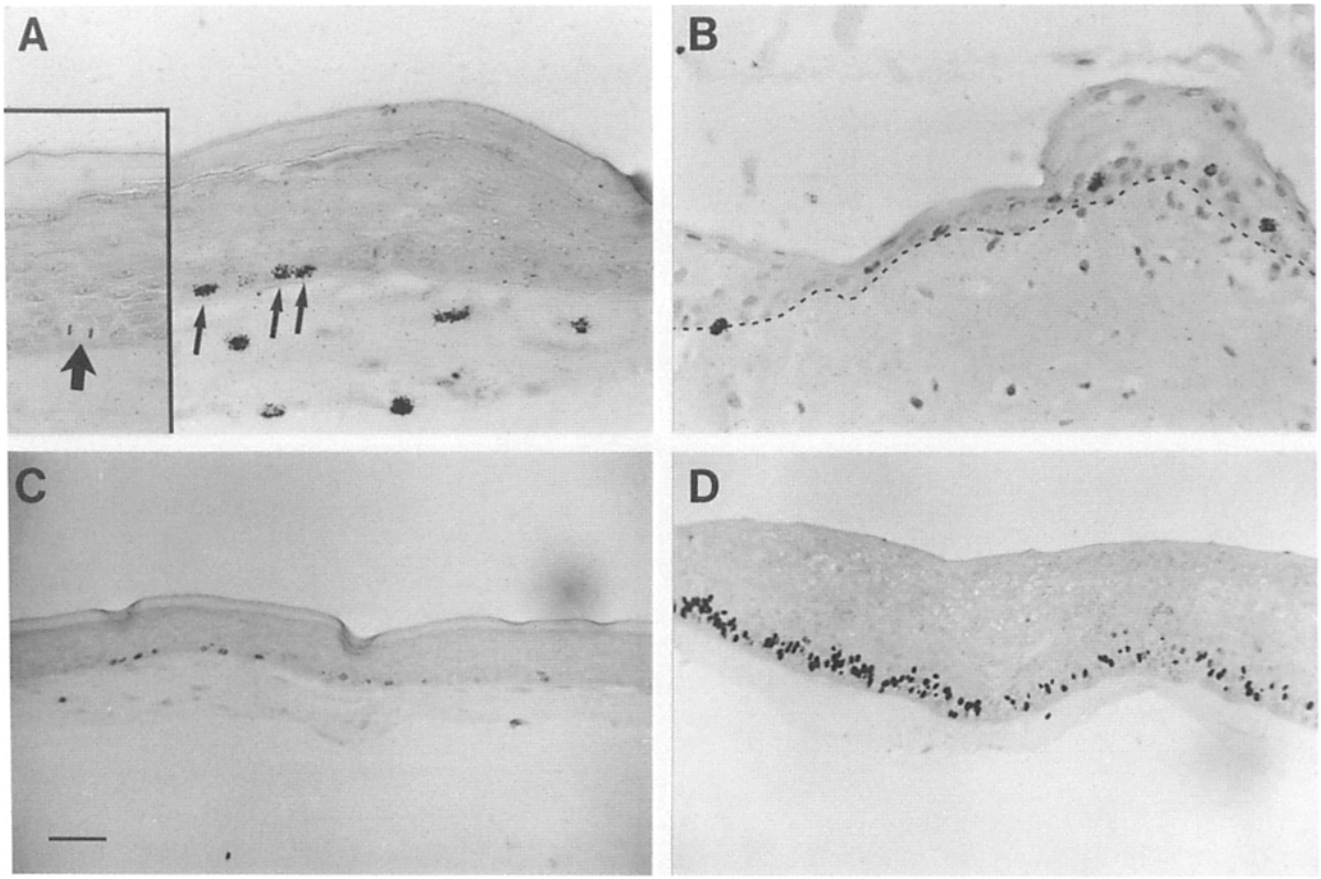

Figure 1. Comparison of morphology and cell proliferation in normal and SCC-13 raft cultures. Human epidermal keratinocytes from normal foreskin and from the squamous cell carcinoma line SCC-13 were cultured on collagen/fibroblast lattices as described in Materials and Methods. Unless otherwise indicated (see below), rafts were floated at the air–liquid interface. 2 h before harvesting, cultures were treated with [^{3}H]thymidine as indicated in Materials and Methods. After radiolabeling, rafts were fixed for 30 min in Carnoy's solution, embedded in paraffin, and sectioned (5 μm). Sections were subjected to autoradiography, followed by staining with hematoxylin and eosin. *A* and *C*, normal epidermal cultures; *B*, normal epidermal culture submerged in medium; *D*, SCC-13 culture. Thin arrows in *A* denote radiolabeling of basal cell nuclei. Thick arrow in the inset of *A* denotes an unlabeled mitotic cell. Note that the basal-preferred labeling pattern is seen in both floating and submerged cultures (*A* and *B*, respectively). Dotted line in *B* denotes demarcation between basal epidermal layer and artificial dermis. Low magnification photographs (*C* and *D*) illustrate the lower proliferation rate in normal versus SCC-13 cultures, respectively. Bar: (*A* and *B*) 30 μm; (*C* and *D*) 74 μm.

BSA in PBS (BSA-PBS). The solution was then replaced with fresh BSA-PBS containing a 1:100 dilution of monospecific rabbit polyclonal antisera against (*a*) a synthetic 7-mer coding for the carboxy terminus of human K6a (anti-K6; antiserum No. 415; Stoler et al., 1989), or (*b*) a synthetic 15-mer coding for the carboxy terminus of human K14 at a 1:200 dilution (anti-K14; antiserum No. 199; Stoler et al., 1988). After a 1-h incubation at room temperature, sections were washed 3× in PBS (10 min each) and then treated with a gold-conjugated goat anti-rabbit antiserum (15-nm gold particles; Janssen Life Science Products, Piscataway, NJ). After an overnight incubation at room temperature, sections were washed 6× in PBS (10 min each), fixed for 15 min in PBS containing 2% glutaraldehyde, and washed 3× in PBS (10 min each) followed by 3× in glass distilled water (10 min each). Sections were then placed in a single glass jar, along with negative control sections in which the primary antiserum was omitted, and positive control sections from skin tissues whose pattern of keratin expression had already been analyzed (Kopan et al., 1987). Sections were then subjected to silver enhancement (IntenSE; Janssen Life Science Products), according to the manufacturer's instructions. Enhancement was stopped at the same time for all sections.

Immunohistochemistry Followed by Autoradiography

Immunohistochemistry was conducted as described above. After stopping the gold enhancement as described by the manufacturer (Janssen Life Science Products), slides were dehydrated via a series of 10-min incubations in increasing ethanol solutions (from 30 to 100%). After air-drying, slides were dipped in NTB2 Kodak emulsion and subjected to autoradiography as described above, with the exception that subsequent staining with hematoxylin and eosin after autoradiography was omitted.

Radiolabeling and Extraction of Proteins

Cultures were labeled for 6 h with 30 μCi/ml [^{35}S]methionine (specific activity 1,000 Ci/mmol; Amersham Corp.). Extraction of intermediate filament proteins was performed using the method previously described by Wu et al. (1982). Rafts were transferred to a Corex centrifuge tube containing 10 mM Tris-HCl (pH 7.3), 0.6 M KCl, 1% Triton X-100, 1.7 mM PMSF, 4°C. Cells were lysed with the aid of sonication, and the insoluble fraction was pelleted by centrifugation at 15,000 *g* for 20 min. The pellet was resuspended in the same buffer, and extracted and centrifuged repeatedly. Intermediate filament proteins were solubilized by dissolving the washed pellet in 8 M urea, containing 10% β-mercaptoethanol.

Immunoblot Analyses

Radiolabeled keratins resolved by SDS-PAGE were transferred to nitrocellulose by electroblotting. Blots were first stained with Ponceau Red (Sigma Chemical Co.) to visualize the transferred proteins. Anti-K6 and anti-K14 antisera were mixed together in a 1:1 ratio and diluted at 1:200 before incubating with the blot (Towbin et al., 1979). Bound antibodies were visualized by secondary antibody binding with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:2,000 dilution), followed by 4-chloro-1-naphthol treatment to develop the color as described by Bio-Rad Laborato-

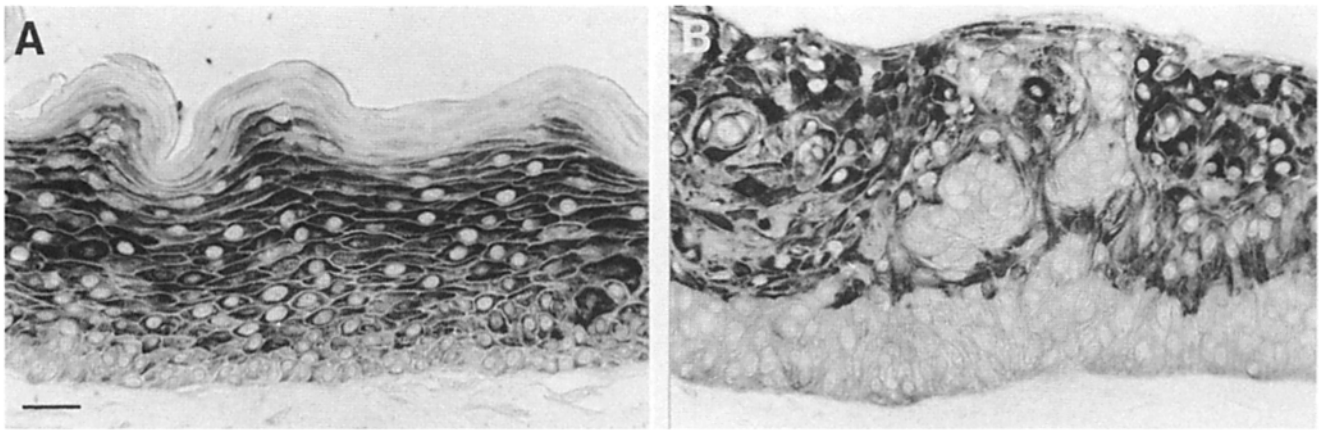

Figure 2. Anti-K6 staining of suprabasal cells of normal and SCC-13 cultures. Epidermal and SCC-13 cells were cultured on rafts at the air-liquid interface. Rafts were fixed in Carnoy's solution for 30 min and then embedded in paraffin and sectioned (5 μm). Sections were deparaffinized and hydrated before exposing them to a 1:100 dilution of a rabbit polyclonal anti-K6 antiserum in 2% BSA in PBS. The antibody was visualized using gold-conjugated goat anti-rabbit IgG antibodies, followed by silver enhancement. *A*, normal epidermal raft; *B*, SCC-13 raft. Note the suprabasal location of anti-K6 staining and the patches of K6-negative cells in the suprabasal layers of SCC-13 cultures. Note: Carnoy's fixation procedure does not interfere with antibody staining for this or other polyclonal keratin peptide antibodies that we have generated (Kopan et al., 1987; Stoler et al., 1988; Stoler et al., 1989). Bar, 30 μm.

ries (Richmond, CA). After photography, immunoblot analysis was then repeated on the same paper, this time using a general anti-type I keratin antiserum (Fuchs and Marchuk, 1983), at 1:50 dilution.

Isolation of Total RNA

Total RNA was isolated from raft cultures according to the method of Chomczynski and Sacchi (1987). Rafts were fed 12 h before harvest. Each raft was solubilized in 1 ml of [4 M guanidinium thiocyanate, 25 mM sodium citrate, pH 7.0, 0.5% Sarkosyl, 0.1 M β-mercaptoethanol]. To each milliliter of cell solution was added, in order, vortexing after each addition: 0.1 ml 2 M sodium acetate, 1 ml phenol, 0.2 ml chloroform. After 10 additional seconds of vortexing, the solution was placed on ice for 15 min. After this incubation, the mixture was subjected to centrifugation at 10,000 *g* for 20 min at 4°C. The upper aqueous phase, containing the RNA, was transferred to a clean tube containing an equal volume of isopropanol. After mixing, the solution was then placed at −20°C for 1 h, followed by centrifugation at 10,000 *g* for 10 min at 4°C. The pellet, containing the RNA, was again extracted in 4 M guanidinium hydrochloride solution, and precipitated with an equal volume of isopropanol as described above. The final pellet was vortexed in the presence of 75% ethanol at room temperature, followed by brief centrifugation, and finally vacuum drying of the pellet. The dried RNA was then dissolved in 0.5% SDS by incubating at 65°C for 10 min.

Northern Blot Analysis

Messenger RNAs were isolated as described above and subjected to Northern blot analyses as described by Thomas (1980). ^{32}P-dCTP-labeled single-stranded cDNA probes were prepared from the following plasmids.

pK5-3'NC. pKA-62 encodes a K5 human cDNA (Lersch and Fuchs, 1988). pK5-3'NC contains the 3' noncoding portion of this K5 cDNA sequence, and it was prepared by subcloning the Nco I/Hae II fragment of pKA-62, extending from 44 nucleotides to 234 nucleotides 3' of the TAA stop codon of the K5 sequence, into the Sma I site of pGEM2 (Promega Biotec, Madison, WI) (Lersch et al., 1989). Antisense probe was prepared by linearizing pK5-3'NC with Hind III and using a T7 RNA polymerase promoter primer (Promega Biotec) and Klenow fragment in the presence of ^{32}P-dCTP and unlabeled deoxyribonucleotides.

K6a-3'NC. pK6a (also referred to as lsp) contains a 1,415-bp Taq I/Sma I fragment of pKA-1 (Hanukoglu and Fuchs, 1983; residues 20–1,435), encompassing the coding portion of K6a, subcloned into the Hinc II site of pSP64. pK6a-3'NC contains a 220-bp Alu I/Spe I fragment extending from 235 bp 3' past the TAA stop codon of the K6a cDNA to 75 bp 5' from the polyadenylation signal, subcloned in the 3' to 5' direction into the Hinc II site of plasmid pGEM1 (Promega Biotec). Antisense probe was prepared by linearizing pK6a-3'NC with Hind III and using a T7 promoter primer and Klenow fragment as described above.

Results

Proliferation Rates Differ for Normal and SCC-13 Cultures, but in Both Cases, DNA Synthesis Is Largely Restricted to the Basal-like Cells

To examine the relative proliferative capacities of the normal epidermal and squamous cell carcinoma raft cultures, we added [^{3}H]thymidine to the medium under the rafts for 2 h before fixation. Cells undergoing DNA synthesis at the time of thymidine treatment incorporated the labeled nucleotide, and the radiolabeled nuclei could be visualized by autoradiography of fixed sections of tissue (Fig. 1). Frame A shows a section (5 μm) of a normal epidermal raft displaying several radiolabeled nuclei in the basal layer (see the three parallel arrows in the frame). After examining many additional sections, we concluded that while the number of cells incorporating label varied considerably throughout the raft, labeling was largely confined to this layer: Only an occasional suprabasal cell showed a radiolabeled nucleus. The mitotically active cells were also predominantly located in the basal layer of the culture. An example of a basal cell fixed during the process of mitosis is shown in the inset of frame A (*thick arrow*).

To test whether the basal epidermal layer might have acted as a barrier to [^{3}H]thymidine, thereby leading to basal-specific radiolabeling of nuclei, we kept an epidermal culture submerged under the medium rather than floating it at the air-liquid interface. Under submerged conditions, stratification was not only reduced in general, but it was also highly variable, with some thicker regions and many poorly stratified regions (see also Kopan et al., 1987). Nevertheless, when a submerged culture was labeled with [^{3}H]thymidine, it showed basal-preferred incorporation of label, even in the thicker regions of the culture (Fig. 1 *B*). Hence, the restriction of labeled nuclei to the basal layer of floating cultures was not simply due to the fact that these cells were closest to the medium containing the label, but rather to an inherent proliferative capacity displayed by the basal population of the

cultures. Collectively, these data are consistent with previous [^{3}H]thymidine studies on human keratinocyte cultures grown on plastic (Milstone and LaVigne, 1985; Albers et al., 1987).

When the squamous cell carcinoma line SCC-13 is cultured on plastic in the presence of a fibroblast feeder layer, it reaches confluence faster than a normal human epidermal culture (Rheinwald and Beckett, 1981). To determine whether SCC-13 cells also proliferate rapidly when cultured on rafts, we repeated our [^{3}H]thymidine experiment using the squamous cell carcinoma line. While the number of cells incorporating labeled nucleotides varied throughout the raft, sections of SCC-13 cultures showed a significantly greater number of cells with radiolabeled nuclei than normal (compare normal [*C*] with SCC-13 [*D*]). Surprisingly, both the [^{3}H]thymidine incorporation and the location of mitotic cells was still predominantly basal, despite the increase in proliferative capacity within the culture. While the percentage of suprabasal cells with radiolabeled nuclei or mitotic figures was somewhat greater than that observed for normal epidermal raft cultures, DNA synthesis and mitosis in the upper layers were still rare events. The increase in cell population generated by this increase in cell proliferation seemed to be accommodated in three distinct ways: (*a*) SCC-13 cultures were more stratified than their normal epidermal counterparts, (*b*) basal cells of the SCC-13 cultures were more compact, forming several layers of cells, and (*c*) the differentiating cells were more crowded than in the normal raft cultures.

Cells That Stain with Antisera against Hyperproliferation-associated Keratins Are Not Synthesizing DNA

Previously, using an antiserum monospecific for K16, we showed that K16 is present in almost all and only the suprabasal cells of normal epidermal raft cultures, while it is patchy, but still suprabasally expressed in SCC-13 cultures (Stoler et al., 1988). To determine whether K6, the partner hyperproliferation-associated keratin, is also expressed suprabasally, we used an antiserum monospecific for K6 (Stoler et al., 1989) and stained sections of our cultures. Fig. 2 shows that similar to anti-K16, anti-K6 stained only the suprabasal, differentiating cells of the normal epidermal cultures (Fig. 2 *A*). The staining pattern was consistent throughout the epidermal population: a single layer of anti-K6-negative basal cells was overlayed with multiple layers of differentiating cells, uniformly staining with the anti-K6 antiserum. The lack of staining in basal cells was even more apparent in SCC-13 cultures, where the basal-like cells were several layers thick (Fig. 2 *B*). Unlike the normal cultures, SCC-13 cultures showed a patchy, suprabasal staining pattern with the anti-K6 antiserum. Interestingly, the same suprabasal regions which showed relatively low or nondetectable levels of anti-K6 staining were also rich with clusters of basal-like cells. Hence, for both cultures, staining with anti-K6 was largely confined to differentiating cells in the suprabasal layers. This result is similar to that obtained with tissue sections of psoriatic epidermis and squamous cell carcinomas (Stoler et al., 1988) and of proliferating epidermis overlying dermatofibromas (Stoler et al., 1989).

The fact that K6/K16 expression was predominantly in differentiating cells and [^{3}H]thymidine labeling and mitotic figures were largely in basal cells suggested that expression of the hyperproliferation-associated keratins and DNA synthesis may be mutually exclusive in the cultures. To answer this question, we took sections (5 μm) of [^{3}H]thymidine-labeled SCC-13 cultures and subjected them first to anti-K6 staining and then to autoradiography. Providing that immunogold labeling and silver enhancement was used as an antiserum

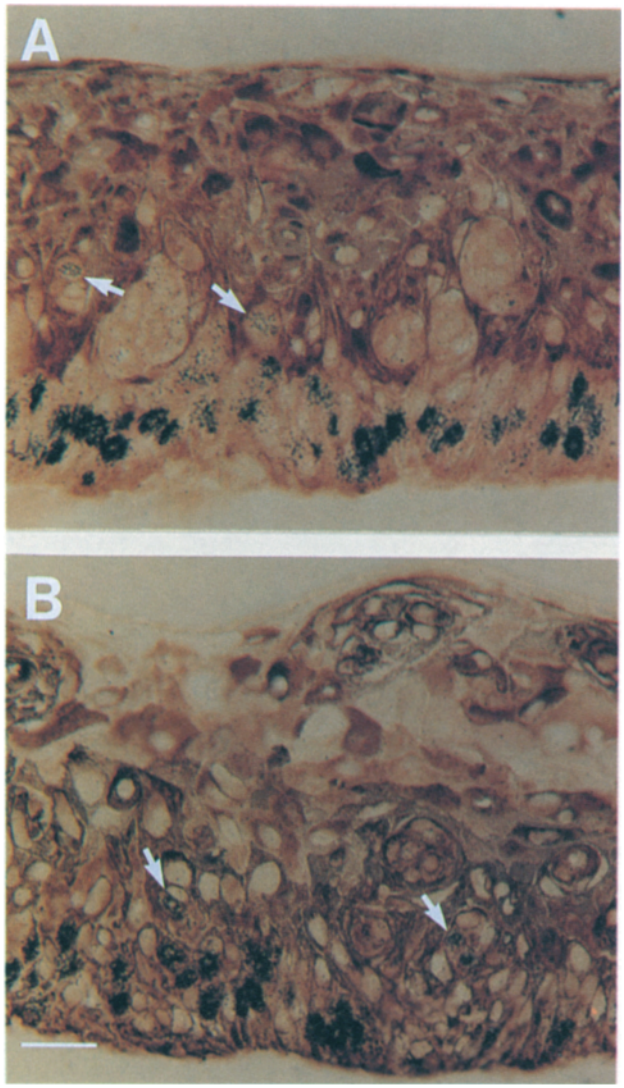

Figure 3. Coupled use of immunogold enhancement and autoradiography to examine keratin expression and cell proliferation in raft sections. SCC-13 cells were cultured for 10 d on floating rafts before [^{3}H]thymidine labeling. After radiolabeling, cultures were fixed, embedded in paraffin and sectioned (5 μm). Sections were hydrated and reacted with one of two antisera: anti-K6, a rabbit polyclonal antiserum monospecific for the carboxy terminal sequence of human K6 (Stoler et al., 1989), and anti-K14, a rabbit polyclonal antiserum monospecific for the carboxy terminal sequence of human K14 (Stoler et al., 1988). Primary antibodies were visualized with gold-conjugated goat anti-rabbit IgG antiserum, followed by silver enhancement. After enhancement, sections were dehydrated in ethanol, air-dried, and subjected to autoradiography as described in Materials and Methods. Note: To prevent interference of the two procedures, it was important to use this method of antibody staining and to conduct the staining before autoradiography. *A*, anti-K6 staining/[^{3}H]thymidine labeling; *B*, anti-K14 staining/[^{3}H]thymidine labeling. Bar, 30 μm.

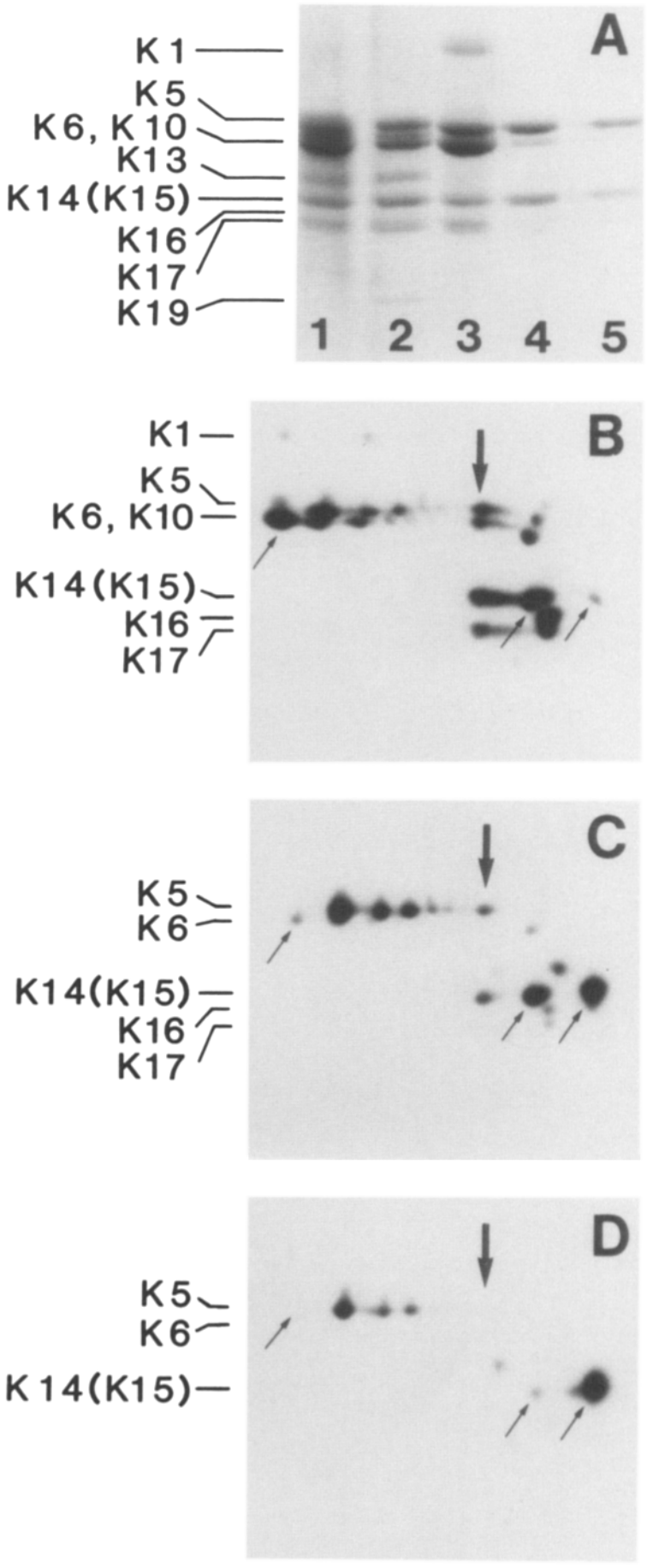

Figure 4. Alterations in keratin patterns produced by retinoid treatments of normal and SCC-13 cultures. Normal and SCC-13 cells were cultured for 10 d on floating rafts over medium containing retinoic acid at a concentration of 0, 1×10^{-7} M, or 1×10^{-6} M. 8 h before harvesting the rafts, [^{35}S]methionine was added at 30 μCi/ml to the culture medium. Keratins were extracted from the epidermal cells as described previously (Fuchs and Green, 1980). Keratins were resolved by one-dimensional gel electrophoresis using SDS polyacrylamide gels (Laemmli, 1970; gel in *A*), or two-dimensional gel electrophoresis using nonequilibrium pH gradient gels in the first dimension and SDS polyacrylamide gels in the second (O'Farrell et al., 1977; gels in *B–D*). After electrophoresis, gels were fixed in methanol/acetic acid solution and subjected to fluorography and autoradiography. Samples in gel in *A* were from: lane *1*, normal epidermal culture; lane *2*, epidermal culture treated with 1×10^{-6} M retinoic acid; lane *3*, SCC-13 culture; lane *4*, SCC-13 culture treated with 1×10^{-7} M retinoic acid; lane *5*, SCC-13 culture treated with 1×10^{-6} M retinoic acid. Samples for two-dimensional gels were from: gel in *B*, SCC-13 cultures; gel in *C*, SCC-13 culture treated with 1×10^{-7} M retinoic acid; gel in *D*, SCC-13 culture treated with 1×10^{-6} M retinoic acid. Keratins are identified according to the nomenclature of Moll et al. (1982). Thick vertical arrow at the top of each two-dimensional gel (*B–D*) denotes complexes between acidic (type I) and basic (type II) keratins which were not resolved in the first dimension NEPHGE gel. From left to right, thin angled arrows point to: K6 (56K), K14 (50K), and a 50-K protein that is more acidic than K14.

staining method, and that the staining was conducted before autoradiography, the two methods were compatible. Fig. 3 *A* revealed that those cells that stained with the anti-K6 antiserum did not incorporate [^{3}H]thymidine, and those cells that incorporated [^{3}H]thymidine did not stain with the anti-K6 antiserum. Even when an occasional suprabasal cell was synthesizing DNA, the cell did not stain with the anti-K6 antiserum (*arrows*, Fig. 3 *A*).

Since the suprabasal cells that incorporated [^{3}H]thymidine were often in clusters of anti-K6-negative regions, and since these cells were usually basal-like in their morphology, we wondered whether these suprabasal patches also had the biochemical characteristics of basal cells. Previously, we showed that the basal layer of normal epidermis stains heavily with a monospecific anti-K14 antiserum (Stoler et al., 1988; Vassar et al., 1989). To determine whether the anti-K6-negative patches of basal-like cells stain prominently with this antiserum, we repeated the staining procedure, this time using anti-K14 before autoradiography. Fig. 3 *B* demonstrates clearly the presence of both anti-K14 staining and [^{3}H]thymidine labeling in a number of basal cells, as well as in the suprabasal patches containing basal-like cells. Thus, suprabasal cells with [^{3}H]thymidine-labeled nuclei were consistently found in patches where anti-K6 staining was weak and anti-K14 staining was strong (*arrows*, Fig. 3, *A* and *B*). Collectively, these data suggest that regions containing suprabasal cells with activity in DNA synthesis and with little or no anti-K6 staining may be less differentiated than suprabasal areas with stronger anti-K6 staining and no detectable [^{3}H]thymidine incorporation.

Inhibition of K6/K16 Expression in Retinoic Acid-treated SCC-13 Cultures

Thus far, our studies suggested a reverse correlation between anti-K6 staining and DNA synthesis. However, previous in vivo studies demonstrated a strong correlation between K6/K16 expression and diseases of epidermal hyperproliferation (Weiss et al., 1983,1984; McGuire et al., 1984; Stoler et al., 1988,1989). One explanation for this apparent discrepancy is that K6/K16 expression may play a role at the tissue level rather than the cell level. Thus, for example, expression of K6 and K16 in suprabasal layers might be necessary to manifest or maintain hyperproliferation in the basal layer below. To determine whether K6/K16 expression might be necessarily coupled to epidermal hyperproliferation, we developed an in vitro method which would enable us to inhibit K6/K16 expression and then assess the consequential effects on cell proliferation. In the course of our investigation, we found several methods which resulted in a reduction in K6 and K16. One of these procedures was to culture SCC-13 cells in the absence of fibroblast feeder layers, a process which resulted in the development of a population of basal-like cells which stratified heavily when cultured at the air-liquid interface, but which did not show signs of differentiation or K6/K16 expression. However, this approach proved to be somewhat variable, and it was not well-defined, particularly since the biochemical nature of fibroblast-epidermal interactions have yet to be elucidated. Hence, we focused our attention towards an alternative method for altering differentiation in culture. In a previous study, we noted that retinoids, whose inhibitory effects on terminal differentiation are well-documented (Fuchs and Green, 1981; Eckert and Green, 1984; Kim et al., 1984; Kopan et al., 1987), have a slight inhibitory effect on K6/K16 expression (Kopan et al., 1987). It was also known that squamous cell carcinoma cells behave as if they were more sensitive to retinoids than normal epidermal cells (Kim et al., 1984). Hence, we investigated the possibility that retinoids might significantly reduce K6/K16 expression in SCC-13 raft cultures.

We first compared the keratin patterns produced by normal and SCC-13 cells cultured on rafts over normal medium, containing serum retinol, but no added retinoids. Both normal and SCC-13 raft cultures expressed K1 (61 kD), K5 (58 kD), K6 (56 kD), K10 (56.5 kD), K11 (56 kD), K14 (50 kD), K16 (48 kD), and K17 (46 kD), many of which were resolved by one-dimensional gel electrophoresis (Fig. 4 *A*, compare lane *1* with *3*). While normal and SCC-13 cultures behaved similarly in normal medium, their different sensitivities to retinoids led to significant differences in keratin patterns when retinoic acid was added to the culture medium. While it is not very apparent in a one-dimensional gel pattern (Fig. 4 *A*, lane *2*), normal epidermal rafts required 1×10^{-6} M retinoic acid to yield a near complete suppression of the terminal differentiation-specific keratins, K1, K10, and K11 (see two-dimensional gel data in Kopan et al., 1987). At this high concentration, retinoic acid also caused a slight down-regulation in K6/K16 levels. Even more striking, however, were the effects of retinoic acid on the expression of K6, K16, and K17 in SCC-13 rafts cultured at the air-liquid interface: at 1×10^{-7} M retinoic acid, these keratins were dramatically down-regulated (Fig. 4 *C*), and at 1×10^{-6} M retinoic acid, these keratins were barely visible (Fig. 4 *D*). Moreover, the K14 spot was markedly reduced, while a new and more acidic spot at 50 kD appeared with increasing intensity (*right arrow*, Fig. 4, *C* and *D*).

To test whether the more acidic 50-kD spot might be a phosphorylated variant of K14, we subjected the radiolabeled keratin extract from Fig. 4 *C* to immunoblot analysis (Fig. 5). Fig. 5 *A* shows the transferred proteins visualized on the blot by Ponceau Red staining. Due to the low sensitivity of the staining procedure, only K5, K14, and the acidic 50-kD spot were visible. Fig. 5 *B* shows the blot after indirect immunoreactivity with a mixture of anti-K6 and anti-K14, followed by a horse peroxidase-conjugated secondary antibody (see Materials and Methods). Under the wash conditions, the Ponceau Red was removed, and now only anti-K6 and anti-K14 cross-reactive spots were visible. These results show that the acidic 50-kD spot was not cross-reactive with the anti-K14 antiserum. To determine whether the acidic 50-kD spot was a keratin, the blot was subjected to a second round of immunoblot analysis, this time using an antisera with broader specificity for most type I keratins (Fuchs and Marchuk, 1983). In this case, the acidic spot was readily detected (*C*). To verify the efficiency of transfer, the blot was subjected to autoradiography (*D*). Thus, the 50-kD protein was a keratin, and it is still possible that the spot was K14, but that a modification in its 15 carboxy terminal amino acid residues prevented its immunoreactivity with the anti-K14 polyclonal antiserum. Further studies will be necessary to determine whether this keratin is a modified K14 or another keratin, e.g., K15.

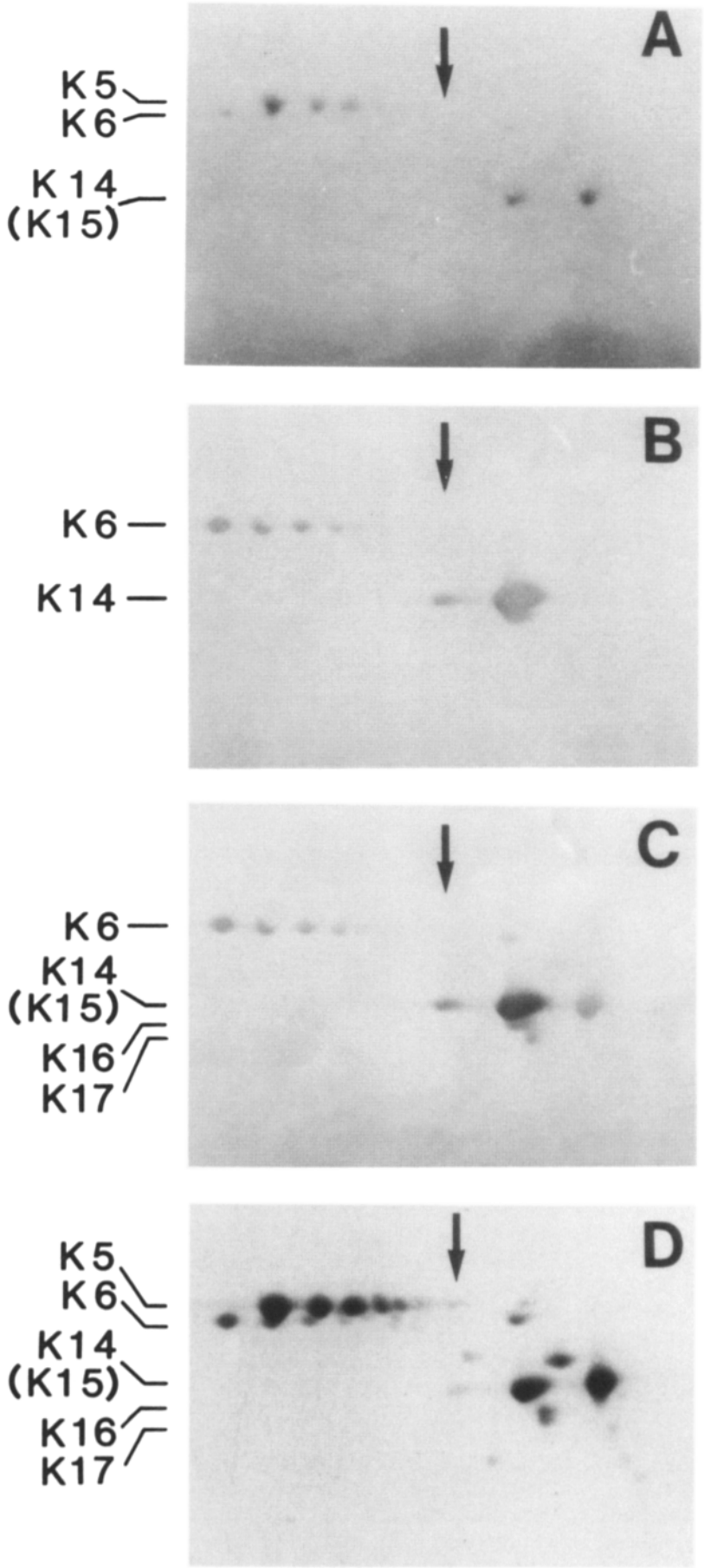

Figure 5. The acidic 50-K protein does not cross-react with anti-K14. The [^{35}S]methionine-labeled keratin extract from SCC-13 rafts cultured in the presence of 1×10^{-7} M retinoic acid was used for analysis (see legend to Fig. 4). Keratins were resolved by two-dimensional NEPHGE gel electrophoresis as indicated in the legend to Fig. 4, and unfixed proteins were transferred to nitrocellulose by immunoblotting (Towbin et al., 1979). The blot was first stained with Ponceau Red to visualize the transferred protein (*A*). The blot was then exposed to anti-K6 and anti-K14, followed by horseradish peroxidase-conjugated secondary antibodies and developing as described in Materials and Methods (*B*). The blot was then blocked and exposed first to a general anti-type I antiserum, followed by a horseradish peroxidase-conjugated secondary antibody (*C*). Finally, the blot was exposed to x-ray film for 24 h before developing (*D*). Keratins are identified at left.

K6/K16 Reduction by Retinoic Acid Is Reflected at the mRNA Level

Previous in vivo studies have detected K6 mRNA, but not protein, in normal human skin (Tyner and Fuchs, 1986; Stoler et al., 1988). However, when skin was placed into culture medium, the level of K6 mRNAs were elevated by 2–6× (Tyner and Fuchs, 1986). Hence, both posttranscriptional and transcriptional controls seemed to be operating to regulate K6 expression. To determine whether the retinoid-mediated suppression of K6 protein expression in vitro is reflected at the mRNA level, we isolated total RNA from normal and SCC-13 rafts cultured in the presence or absence of retinoic acid. RNAs (2 μg each) were resolved by formaldehyde agarose gel electrophoresis and transferred to nitrocellulose paper by blotting (Thomas et al., 1980). Duplicate blots were then hybridized with two different ^{32}P-radiolabeled probes specific for the 3′ noncoding portions of K5 and K6a mRNAs, respectively.

The results of our Northern blot analyses are shown in Fig. 6. To compare the level of K6 mRNA relative to the level of K5 mRNA, each lane hybridized with the K5 probe (*left section*) was exposed for the same amount of time as the corresponding lane hybridized with the K6 probe (*right section*). Relative to K5 mRNA, the level of K6 mRNA did not change appreciably when normal epidermal rafts were cultured in the presence of retinoic acid (lanes *1* and *2*, compare *left* and *right sections*). In contrast, when the SCC-13 cultures were treated with retinoic acid, the level of K6 mRNAs began to decline relative to K5 mRNA (lanes *3–5*, compare *left* and *right sections*). This decline was evident at 1×10^{-7} M retinoic acid (lane *4*, *right section*), and it was even more prominent at 1×10^{-6} M retinoic acid (lane *5*, *right section*). The retinoid-mediated decline in K6 mRNA relative to K5 mRNA in SCC-13 cells paralleled the protein data provided in Fig. 4, and hence, much of the control in K6 expression seemed to be exerted at the mRNA level. However, when 1×10^{-6} M retinoic acid was present in the culture medium, K6 mRNA was still detected, even though the expression of K6 protein was almost completely inhibited. Thus, while most of the regulation in retinoid-mediated reduction in K6 was at the mRNA level, there seemed to be a low level of post-mRNA control.

Reduction in K6 and K16 in Retinoic Acid–treated SCC-13 Rafts Is Not Accompanied by a Decrease in Cell Proliferation

A priori, it seemed possible that expression of K6 and K16 in suprabasal cells and hyperproliferation in basal cells might be necessarily linked. Since retinoic acid completely suppressed K6/K16 expression in SCC-13 raft cultures, we wondered whether retinoic acid might also lead to a decrease in the proliferation of the basal-like cells. To answer this question, we examined DNA synthesis as a function of increasing concentrations of retinoic acid in a range from 0 to 1×10^{-6} M (Fig. 7). Surprisingly, the number of cells incorporating [^{3}H]thymidine showed no decline with increasing concentrations of retinoic acid, and if anything, the percentage of incorporating cells increased (Fig. 7, *A–C*, respectively). Stratification of the retinoid-treated cultures decreased, and the remaining four to seven layers of cells were basal-like in morphology. DNA synthesis often extended into these suprabasal layers of retinoid-treated cultures (*B* and *C*). The retinoid-mediated inhibition of differentiation also occurred in normal epidermal cultures, although it was not as pronounced as in the SCC-13 cultures (see Kopan et al., 1987 for retinoid studies on normal cultures). As expected from the protein data (Fig. 4), the retinoid-treated SCC-13 cultures showed a marked decline in anti-K6 staining (*D–F*).

Collectively, our data reveal an uncoupling between K6/K16 expression and epidermal hyperproliferation: in SCC-13 cells, retinoic acid suppressed the processes of both normal (K1/K10) and abnormal (K6/K16) differentiation without decreasing proliferation in the basal-like population.

Discussion

Epidermal Proliferation and K6/K16 Expression

A number of studies have demonstrated the abnormal expression of K6 and K16 in human diseases of the epidermis (Baden et al., 1978; Hunter and Skerrow, 1981; Thaler et al., 1980; Breitkreutz et al., 1981; Weiss et al., 1983,1984; Moll et al., 1983,1984; McGuire et al., 1984; Leigh et al., 1985; Stoler et al., 1988,1989). While these keratins are not normally expressed in epidermis, they are induced transiently

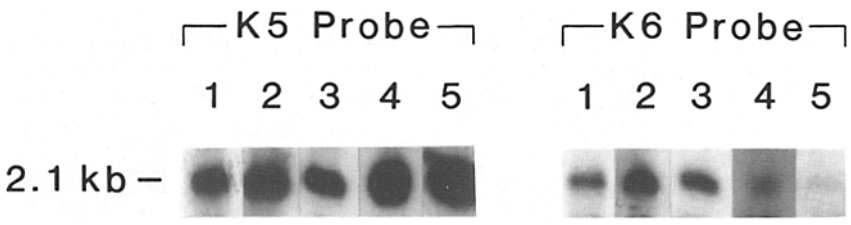

Figure 6. Northern blot analyses to examine the effects of retinoic acid on keratin mRNA patterns. Normal epidermal and SCC-13 rafts were cultured at the air-liquid interface over medium containing 0, 1×10^{-7}, or 1×10^{-6} M retinoic acid. After 10 d, a raft from each condition was harvested for RNA extraction (Chomcyznski et al., 1979). Total RNAs (2 μg) were loaded in duplicate and subjected to electrophoresis through formaldehyde agarose gels. RNAs were transferred to nitrocellulose by blotting (Thomas et al., 1980), and one blot was hybridized with radiolabeled cDNA probe to the 3′ noncoding portion of K5 mRNA (*left*), while a duplicate blot was hybridized with radiolabeled cDNA probes to the 3′ noncoding portions of K6 mRNA (*right*). Blots were exposed to x-ray film for 24 h, 3 d, and 7 d. Lanes were chosen such that the amount of signal corresponding to the K5 mRNA band was approximately equal for each condition. For each lane shown in the left blot, the corresponding lane in the right blot was exposed for exactly the same time. Note that the difference in background of lane 4 (K5) and lane 4 (K6) was due to a light leak rather than to different exposure times. Sizes were determined by molecular weight RNA markers and are indicated at the left in kilobase pairs.

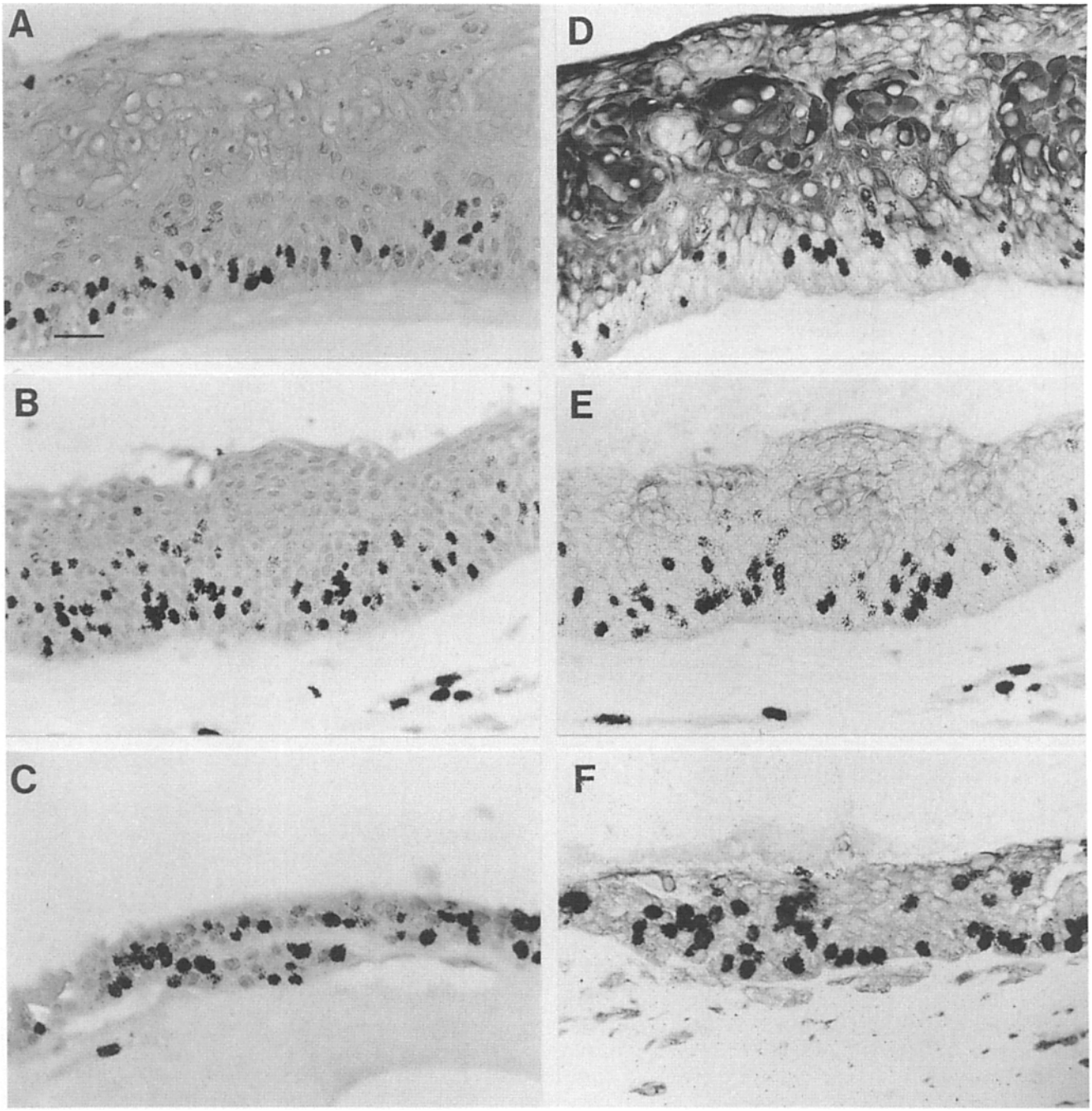

Figure 7. Effects of retinoic acid on cell proliferation and anti-K6 staining of SCC-13 raft cultures. Additional details of these procedures may be obtained in Materials and Methods. SCC-13 rafts were cultured at the air–liquid interface over medium containing 0 (*A* and *D*), 1×10^{-7} M, (*B* and *E*), or 1×10^{-6} M (*C* and *F*) retinoic acid. After 10 d, rafts were labeled with [^{3}H]thymidine for 2 h and then fixed, embedded in paraffin, and sectioned (5 μm). Sections were either subjected to autoradiography, followed by hematoxylin, and eosin staining (*A–C*), or subjected to immunohistochemistry with anti-K6 antiserum (*D–F*). Bar, 30 μm.

during wound healing (Lane et al., 1985; Mansbridge and Knapp, 1987), or when skin is placed into tissue culture medium in vitro (Sun and Green, 1978; Fuchs and Green, 1980; Tyner and Fuchs, 1986). Under all of these conditions, the expression of K6 and K16 seems to be associated with an increase in epidermal proliferation, and it has been suggested that the keratin pair may be a biochemical marker for hyperproliferating keratinocytes (Weiss et al., 1984).

Previously we showed that K6 and K16 are expressed in the suprabasal cells of psoriatic epidermis and squamous cell carcinoma, concomitantly with a down-regulation of the terminal differentiation-specific keratins (K1 and K10) (Stoler et al., 1988). The shift from the expression of one set of keratins to another in the suprabasal layers was suggestive that the differentiative process was altered in these two diseases of epidermal proliferation. However, since epidermal prolif-

eration is often associated with an extension of proliferating cells into the suprabasal layers (Lavker and Sun, 1983; Weinstein and Van Scott, 1965; Leigh et al., 1985), it was not clear from these early studies whether K6/K16 was expressed in mitotically active cells which had undergone an increase in number, or whether K6/K16 was expressed in differentiating, nondividing cells which were derived from a population of basal cells which had undergone an increase in proliferation.

Our use of immunohistochemistry coupled with [^{3}H]thymidine labeling left little doubt that the expression of K6/K16 and DNA synthesis were mutually exclusive in our in vitro cultures. Thus, it seems likely that the abnormal synthesis of these keratins in some proliferative diseases also occurs predominantly in the differentiating, rather than the dividing, cells of the population. Moreover, our discovery that retinoic acid inhibits K6 and K16 expression in SCC-13 raft cultures and simultaneously gives rise to an increase in the number of proliferating (i.e., basal-like) cells within the population argues strongly that the frequent association of these keratins with the hyperproliferative state is more likely to be a consequence, rather than a cause of increased epidermal proliferation.

While our results demonstrate that K6/K16 do not seem to play an essential role in promoting epidermal proliferation, this does not necessarily imply that the expression of these keratins is irrelevant in contributing to the disease state. In vitro assembly studies have shown that K6 and K16 can each coassemble separately with epidermal keratins into 8-nm filaments (Hatzfeld and Franke, 1985). In vivo studies involving transfection of K6 and K16 genes into tissue culture cells have confirmed that each of these keratins incorporates into the existing and growing keratin filament network (Giudice and Fuchs, 1987; Rosenberg et al., 1988). Thus, K6- and K16-containing 8-nm filaments probably exist within the keratin networks of differentiating cells of diseases such as psoriasis or squamous cell carcinomas. It is possible that the substitution of K6/K16-containing filaments for K1/K10-containing filaments might alter the properties of the cytoskeleton of a cell. Alternatively, such changes could alter the intercellular interactions within the sheets of suprabasal cells. In this regard, recent gene transfection studies have revealed that alterations of the cytoskeleton within one cell can influence the intercellular interactions and the cytoskeletons within adjacent cells (Albers and Fuchs, 1989). Although intercellular interactions are known to be affected in scaling diseases such as psoriasis, it remains to be tested whether K6/K16 expression has a functional role in this or any other feature of abnormal differentiation.

Retinoids and Their Effects on K6/K16 Expression and DNA Synthesis: How Might These Effects Be Physiologically or Clinically Relevant?

Since the early studies of vitamin deficiency (Mori, 1922; Wolbach and Howe, 1925) and vitamin excess (Fell and Melanby, 1953), vitamin A has been known to have a pronounced negative affect on epidermal differentiation. Early cell culture studies reported morphological changes in keratinocytes treated with varying amounts of retinoids (Yuspa and Harris, 1974; DeLuca and Yuspa, 1974; Sporn et al., 1975; Elias et al., 1981). More recently, it was found that retinoids at a concentration of ~10× higher than physiological can suppress expression of terminal differentiation-specific keratins (K1 and K10) (Fuchs and Green, 1981; Kim et al., 1984; Eckert and Green, 1984; Gilfix and Green, 1984; Kopan et al., 1987) as well as proteins involved in cross-linked envelope formation (Green and Watt, 1982). While most biochemical studies on the effects of retinoids have been conducted with keratinocytes cultured on plastic, it has recently been demonstrated that retinoids added to the culture medium of epidermal cultures floating on rafts of collagen and fibroblasts can inhibit the biochemistry and the morphology of the extensive terminal differentiation process which takes place in these cultures (Kopan et al., 1987). Since in vivo, blood vessels supplying vitamin A to skin are located in the dermis, it has been suggested that terminal differentiation both in vitro and in vivo may be programmed by progressive migration of differentiating epidermal cells away from the source of retinoids (Kopan et al., 1987).

A priori, since retinoic acid was known to suppress K1/K10 expression, and since K6/K16 expression was typically elevated in epidermal cells when K1/K10 expression was suppressed, the retinoid might have been expected to enhance, rather than suppress K6/K16 expression. The fact that both sets of suprabasal keratins are suppressed by retinoic acid suggests that the retinoid has a negative effect on both normal and abnormal forms of epidermal differentiation. We do not yet know why retinoic acid quantitatively inhibited the expression of K6/K16 only in SCC-13 raft cultures, and not in normal epidermal raft cultures. However, previous studies by Kim et al. (1984) have shown that when cultured on plastic, squamous cell carcinoma cells of epidermal origin (Wu and Rheinwald, 1981), behaved as if they had an internal retinoid concentration ~10× higher than normal epidermal cells. Hence, it may be that the phenomenon depends on the degree to which an epidermal cell is sensitive to retinoids. If so, at physiological concentrations of retinoids, the suppression of K6/K16 may only be manifested in epidermal cells that have undergone some biochemical change, e.g., one similar to that which occurs in malignant transformation or psoriasis.

The suppression of K6/K16 in retinoid-treated cultures of SCC-13 but not normal epidermal cells may also be related to morphological differences which were observed between the two cell cultures: SCC-13 cultures treated with retinoic acid appeared undifferentiated, with multiple layers of basal-like cells. This morphology was also observed for feeder-independent SCC-13 cultures, which also showed inhibition of K6/K16 (Kopan, R., D. McCance, L. Laimins, and E. Fuchs, unpublished observations). In contrast to SCC-13 cultures, epidermal cultures treated with retinoic acid retained a gradient of differentiated cells, similar to that seen in vitro for untreated SCC-13 cultures and in vivo for patients suffering from hypervitaminosis A (see also Kopan et al., 1987). While the basal-like appearance of the retinoid-treated or feeder-independent SCC-13 cultures may in part explain why they did not show K6/K16 expression, the phenomenon is clearly a more complex one: SCC-13 cultures kept submerged under medium containing up to 1×10^{-6} M retinoic acid also appeared basal-like and yet still expressed K6 and K16, as did submerged SCC-13 cultures from feeder-independent lines (Kopan, R., and E. Fuchs, unpublished observations). Thus, unlike retinoid-mediated suppression of K1/K10, suppression of K6/K16 in SCC-13 cultures seemed

to be dependent upon the polarity established by growing the raft at an air–liquid interface.

In summary, our studies have demonstrated that low levels of retinoids have a profound effect on enhancing the population of basal-like, thymidine-incorporating cells of both normal and squamous cell carcinoma cells cultured on floating rafts at an air–liquid interface. Concomitantly, while retinoids suppress the expression of terminal differentiation-associated keratins in both normal and malignant cells, they quantitatively suppress the expression of the hyperproliferation-associated keratins only in the tumor cells, which seem to have ~10× greater sensitivity to retinoids than normal keratinocytes. This inhibition of K6/K16 seems to be dependent on the polarity of the culture with respect to its media source, and occurs simultaneously with a loss of differentiated cell morphology. While the functional significance of the abnormal expression of K6/K16 remains unknown, the process of K6/K16 expression and epidermal proliferation can be uncoupled, suggesting that these keratins may be a frequent consequence, rather than a cause, of the hyperproliferative state.

Retinoids are commonly used in the clinical treatment of many different skin disorders. Our finding that retinoids can inhibit both the expression of K6/K16 and also the abnormal differentiation typically associated with hyperproliferation might explain some of the therapeutic effects of vitamin A and its analogues on the skin. However, the finding that retinoids can increase the population of basal-like cells without a loss in their proliferative potential may be an alarming side effect of the successful inhibition of abnormal differentiation.

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