UC Davis
UC Davis Previously Published Works

Title

Rat esophageal and epidermal keratinocytes: Intrinsic differences in culture and derivation of continuous lines

Permalink

https://escholarship.org/uc/item/08m948q0

Journal

Journal of Cellular Physiology, 117(3)

ISSN

0021-9541

Authors

Heimann, Ruth
Rice, Robert H

Publication Date

1983-12-01

DOI

10.1002/jcp.1041170311

Peer reviewed

eScholarship.org Powered by the California Digital Library
University of California

JOURNAL OF CELLULAR PHYSIOLOGY 117:362–367 (1983)

Rat Esophageal and Epidermal Keratinocytes: Intrinsic Differences in Culture and Derivation of Continuous Lines

RUTH HEIMANN AND ROBERT H. RICE*
Charles A. Dana Laboratory of Toxicology, Harvard School of Public Health, Boston, Massachusetts 02115

Serially cultivated with 3T3 feeder layer support as colonies of stratified squamous epithelium, rat epidermal and esophageal epithelial cells were readily distinguishable by three critera. First, the epidermal colonies, exhibiting extensive piling up of squames in the centers, were more stratified than esophageal colonies. Second, in sparse culture 70 to 90% of the esophageal cells but as few as 1 to 5% of the epidermal cells were competent in cross-linked envelope formation upon treatment with the ionophore X537A. After reaching confluence, up to 90% of the cells of both types formed envelopes upon ionophore treatment. Third, epidermal cells in suspension culture reached maximal levels of spontaneously cross-linked envelopes in 1 day or less, while esophageal cells required about 4 days in suspension to reach maximal levels. A reproducible finding with both cell types was that initial colony-forming efficiencies of less than 1% increased to about 40% upon serial passage with consequent derivation of continuous lines. Sparse cultures of esophageal cells with high colony-forming ability retained a high degree of envelope competence (70 to 90%), indicating these two properties are not mutually exclusive. The derived lines exhibited reduced dependence upon feeder layer support at clonal density, but in suspension culture the cells did not grow and lost colony-forming ability with a half-time of several hours. We conclude that cells from these keratinized rat epithelia exhibit intrinsic differences in culture and become continuous lines expressing characteristic regulation of envelope competence and loss of germinative capability in suspension.

Stratified squamous epithelia at various anatomical sites (e.g., epidermis, esophagus, exocervix) arise from different embryonic germ layers and hence through separate developmental pathways. The constituent keratinocytes exhibit a common program of terminal differentiation in which a high content of keratin tonofilaments and synthesis of cross-linked protein envelopes comprise two distinctive biochemical features (Green, 1980). Such epithelia differ biochemically in gel electrophoretic pattern of keratin polypetides (Moll et al., 1982; Wu et al., 1982) and histologically (depending upon the species) in expression of a superficial layer of enucleated squames (*S. corneum*). According to transplantation experiments, this incomplete convergence of differentiated properties evidently reflects intrinsic differences within the epithelial cells rather than specific mesenchymal instruction (Doran et al., 1980), although modulating influences of factors such as vitamin A are demonstrable (Fuchs and Green, 1981). The physiological and molecular bases for differential regulation of properties shared by cells of this type remain to be characterized further.

Methods now exist for the serial cultivation of keratinocytes with feeder layer support from lethally irradiated 3T3 cells (Rheinwald, 1980). The growing cultures consist of tightly packed stratified colonies (Rheinwald and Green, 1975) in which the cells have a high keratin content (Sun and Green, 1978) and are capable of expressing the features of terminal differentiation observed in vivo (Green, 1977). Although relatively few normal human epidermal cells form cross-linked envelopes in surface culture, a large majority will do so when their membranes are made permeable to calcium ion, activating the cellular transglutaminase (Rice and Green, 1979). In suspension culture, the normal keratinocytes rapidly lose germinative capability and spontaneously form envelopes (Green, 1977), a process in which continuous lines derived from human squamous cell car-

Received March 16, 1983; accepted July 26, 1983.

Ruth Heimann is now at the Department of Pharmacology, New York University Medical Center, 550 First Avenue, New York, NY 10016.

*To whom reprint requests/correspondence should be addressed.

© 1983 ALAN R. LISS, INC.

cinomas are defective (Rheinwald and Beckett, 1980). The present work explores intrinsic differences in cultured rat epidermal and esophageal epithelial cells, emphasizing regulation of cross-linked envelope formation. We also report that primary cultures derived from rat tissue routinely become continuous lines upon serial passage.

MATERIALS AND METHODS

The epithelial cells were cultivated with feeder layers of lethally irradiated mouse 3T3 cells according to standard procedures (Rheinwald and Green, 1975; Rheinwald, 1980). Primary cultures were initiated with trypsin-disaggregated cells from minced tissue of adult Sprague-Dawley rats (Charles River Laboratories). Dulbecco-Vogt Eagle's growth medium was supplemented with fetal bovine serum, hydrocortisone (0.4 μg/ml), cholera toxin (9 ng/ml), and epidermal growth factor (10 ng/ml). For routine growth in early experiments, the concentration of fetal bovine serum was 20% for esophageal and 5% for epidermal cultures; later experiments showed that the serum concentration had no effect on the intrinsic differences observed, and both cell types were grown with 5% serum. Contaminating tissue fibroblasts were removed periodically as necessary by spraying with 0.02% EDTA in isotonic saline (Sun and Green, 1976) and disappeared after several passages. With serial propagation at densities of 500–1000 colonies per dish, each passage represented approximately ten generations, as described for human epidermal cells (Rheinwald and Green, 1975).

Colony-forming efficiencies were estimated by number of macroscopic colonies (at least 50 cells) in formalin-fixed cultures stained with Rhodanile Blue. For determination of survival in suspension culture, cells were suspended at 10^5/ml in medium containing 1.3% methylcellulose (Dow A-4 M) supplemented with 20% fetal bovine serum. After various periods of incubation they were recovered by tenfold dilution in medium with subsequent centrifugation, and colony-forming efficiency was measured (Green, 1977). Competence to form cross-linked envelopes was determined by suspending the cells (8×10^5/ml) in serum-free medium containing X537A (50 μg/ml) for 2 hr at 37° (Rice and Green, 1979). Approximately 20 min after adding sodium dodecyl sulfate to 2% and dithioerythritol to 20 mM, envelopes were counted (usually 200 to 400) by phase-contrast microscopy. Keratin polypeptides were prepared by extraction of homogenized cells or tissue twice in 2% Triton X-100–10 mM sodium phosphate buffer (pH 7.2) and then twice in this Triton–phosphate buffer adjusted to 0.45 M in NaCl. The insoluble residue was rinsed once in water, dissolved by boiling in 2% sodium dodecyl sulfate–20 mM dithioerythritol–5 mM sodium phosphate buffer (pH 7.2), and submitted to electrophoresis in 7.5% polyacrylamide slab gels (Laemmli, 1970).

RESULTS

As illustrated in Figure 1, disaggregated cells from rat epidermis and esophagus initiated tightly packed epithelial colonies that pushed back the feeder layer as they expanded. Some esophageal colonies appeared stratified early, but most did not exhibit visible squame formation until reaching moderate size (200 cells), at which time stratification (Fig. 1b) became visible first in the center. The epidermal colonies were more stratified than the esophageal. The extensive piling up of cells in early passages is pictured microscopically in Figure 1c and was often apparent macroscopically in living cultures. As has been generally found with keratinocytes,

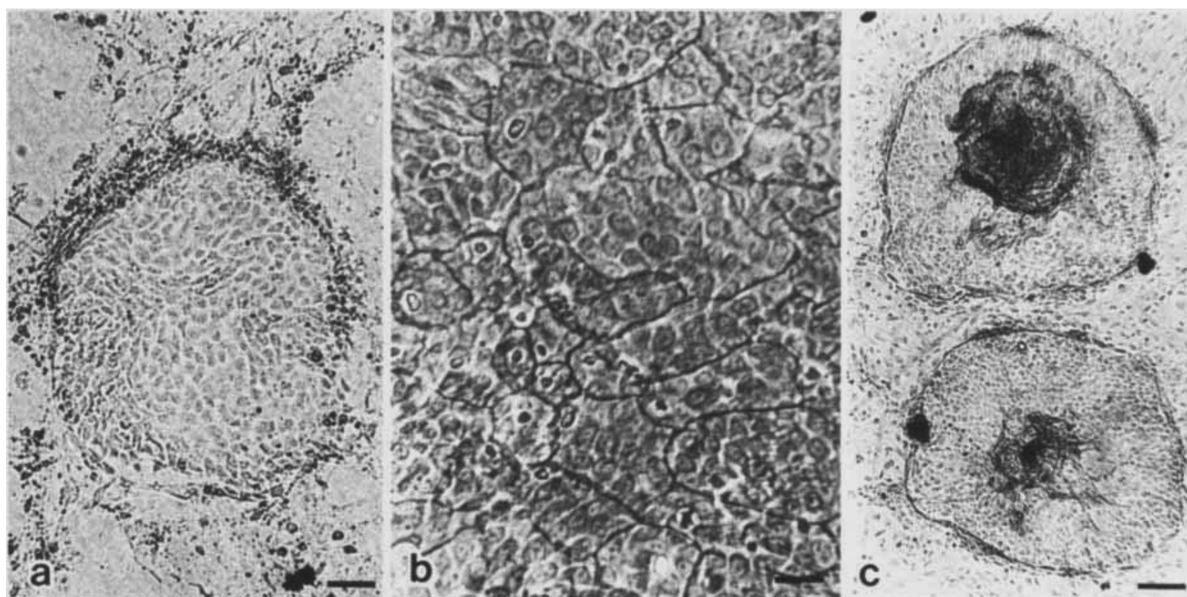

Fig. 1. Keratinocyte colonies derived from rat esophagus (rEs) and epidermis (rEp). The epidermal colonies (c) at early passage are clearly more highly stratified than the esophageal colonies (a), although the latter exhibit stratification in the centers when large (b). Large flattened squames with dark margins cover the small cells of the basal layer (b). Bar: 50 μm in (a), 10 μm in (b), 100 μm in (c).

colony size of both cell types increased faster when they were grown in the presence of hydrocortisone (Rheinwald and Green, 1975), epidermal growth factor (Rheinwald and Green, 1977), and cholera toxin (Green, 1978).

Derivation of continuous lines

Colony-forming efficiencies were 0.01 to 0.1% in primary cultures and several percent in early passages. As shown in Figure 2, these values increased considerably upon continued passage. In three separate experiments, the esophageal cells reached a maximum of 40 to 50% efficiency in about 10 to 12 passages, while epidermal cells from two samples required 17 to 20 passages to reach a maximum. At these later passages, the epidermal cultures did not exhibit such enhanced stratification as in early passage. Although some random fluctuations in the measured efficiencies were observed, probably reflecting the small numbers of cells inoculated per dish and their tendency to aggregate, the cells showed no tendency to senesce or to experience a period of crisis, unlike mouse embryo cells (Todaro and Green, 1963).

The observed increase in colony-forming efficiency evidently signified establishment of continuous lines, at which time colony sizes increased almost as fast without as with added cholera toxin and epidermal growth factor. Regardless of these additions, colony formation at clonal densities ($< 10^3$ cells/60-mm dish) was completely dependent upon feeder layer support for at least several more passages. Eventually, the dependence upon feeder support became less stringent, as shown in Figure 2, with colony-forming efficiencies usually in the range of 10 to 20%. At high density (10^5/dish), the cells grew well and passaged readily without a feeder layer. At low density (10^2/dish), the colonies grew slowly, but progressively, and were greatly stimulated by irradiated 3T3 cells even after several passages in the absence of feeders.

Cross-linked envelopes and keratins

Both esophageal and epidermal cells from confluent cultures were highly competent to form cross-linked envelopes upon treatment with the ionophore X537A. This capability was tested as a function of degree of confluence, as shown in Figure 3. Within several days of seeding, at which time colonies consisted of only several cells each, esophageal cultures were nearly as competent as at confluence, while epidermal cultures by contrast exhibited very low competence (5% or less). This phenomenon was observed in early passage and after establishment of continuous lines and was not affected by serum concentration in the medium (tested in the range of 5 to 20%).

Envelope cross-linking without ionophore treatment occurs spontaneously in keratinocytes in suspension culture (Green, 1977). As shown in Table 1, the two rat epithelial cell types formed envelopes spontaneously at considerably different rates in suspension. The epidermal cells reached maximal envelope levels within 1 day (no ionophore treatment), while the esophageal cells showed only a slightly higher percentage after 1 day than at the time of suspension, and required about 4 days to reach maximal levels. With both cell types, however, the final percentages attained even with ionophore treatment were significantly less than the degree of competence measured with ionophore when the cells were first put into suspension. Envelopes did not seem to be lost by continuing degradative processes, since prolonged suspension for up to 13 days did not reduce the percentage further. Rather, envelope competence itself dropped during the first day. As shown in Table 1, these effects were observed using cells of early and later passage, although in repeated experiments the epidermal cells of late passage exhibited somewhat reduced levels of ionophore-inducible envelopes.

Figure 4 shows the gel electrophoretic band patterns of the aqueous-insoluble keratin proteins isolated from rat esophagus and epidermis and from the corresponding cultured epithelial cells. It is clear that the cultured cells express keratins in the 40,000 to 55,000 dalton

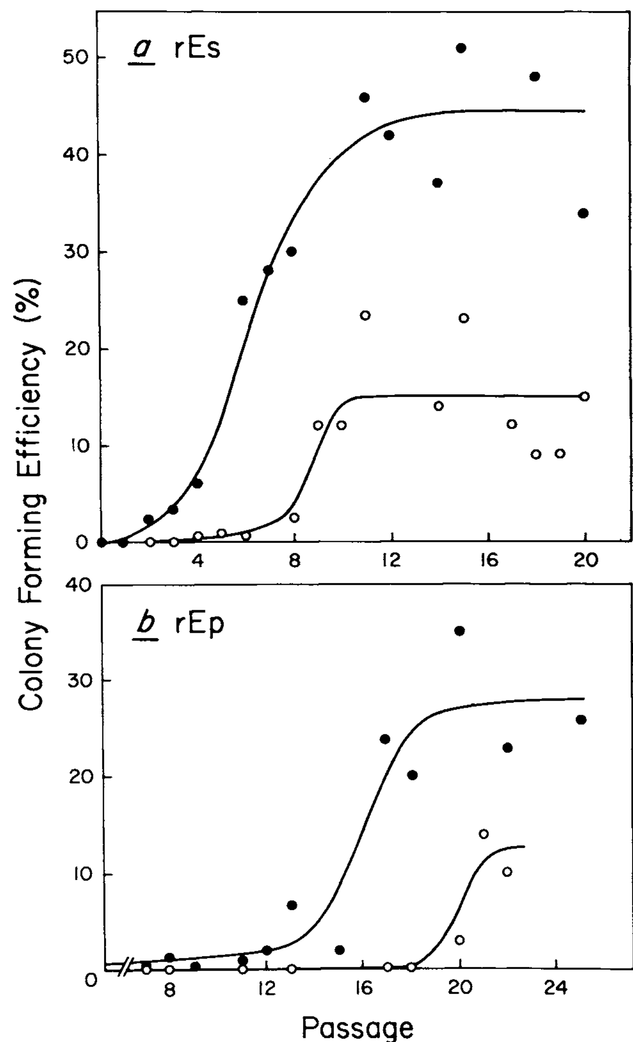

Fig. 2. Colony-forming efficiency as a function of passage at clonal density in the presence (●) or absence (○) of a 3T3 feeder layer. Cell populations were maintained and passaged in the presence of the feeder layer with periodic sampling for colony-forming efficiency in the presence or absence of the 3T3. In these experiments, the esophageal (rEs) cells (a) reached maximum colony-forming efficiency in 10 to 12 passages, while the epidermal (rEp) cells (b) required 17 to 20 passages.

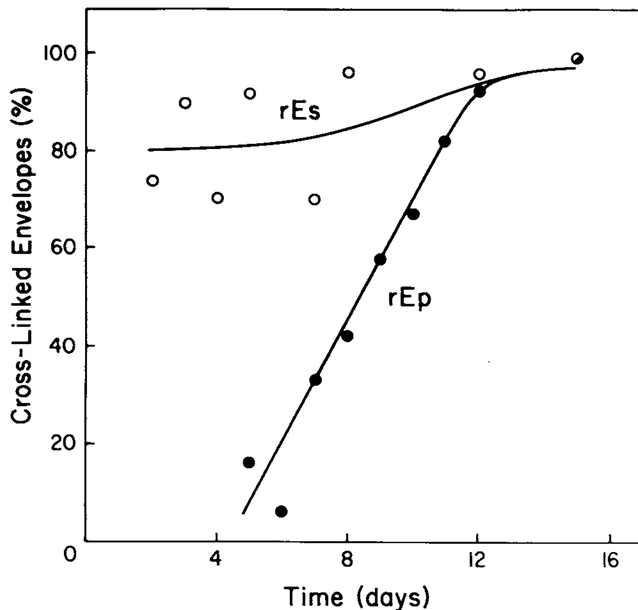

Fig. 3. Dependence of ionophore-assisted envelope formation on culture density. The rat esophageal (rEs) cells (○) were highly capable of envelope formation in sparse and dense culture, while the rat epidermal (rEp) cells (●), exhibited considerable envelope formation only as the cultures approached and remained at confluence. Pooled data of several experiments are presented in which each value is the average of at least two dishes. Replicate dishes generally agreed within 10%. In the absence of added ionophore, the percentage of cells spontaneously forming envelopes in surface culture was 5 to 10% of the total envelopes.

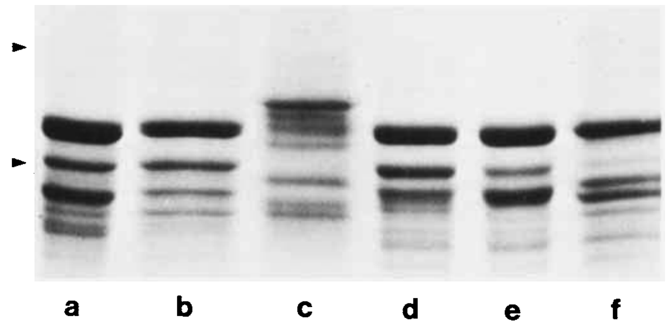

Fig. 4. Keratin patterns from rat epidermis (rEp) and esophageal (rEs) epithelium and the corresponding cultured cells. Samples in different lanes are (a) rEp_{22}; (b) rEp_2 (rat epidermal cells of passages 22 and 2, respectively); (c) rat epidermis; (d) rEs_{19}; (e) rEs_5 (rat esophageal cells of passages 19 and 5); and (f) rat esophageal epithelium. Migration of the molecular weight standard proteins bovine serum albumin (68,000 daltons) and glial filament protein (50,000 daltons) are indicated on the left by the upper and lower arrowheads, respectively.

TABLE 1. Spontaneous envelope cross-linking and competence in suspended epidermal and esophageal keratinocytes[1]

Cell type	X537A	Days 0	1	4
rEp_5	−	7	55	57
	+	100	54	56
rEs_5	−	5	14	50
	+	99	42	54
rEp_{21}	−	5	24	30
	+	49	28	31
rEs_{18}	−	4	9	28
	+	96	33	33

[1]After recovery of the suspended cells, spontaneous cross-linking and competence, respectively, were scored on aliquots without (−) and with (+) treatment with the ionophore X537A. Rat esophageal (rEs) and epidermal (rEp) cultures were employed at passage numbers indicated by subscripts.

range expected for this class of protein, and that the pattern for each epithelial type changes little as continuous lines are established by serial passage. The keratin patterns from the two epithelial lines are rather similar to each other. In contrast, the keratins produced in vivo by these epithelia exhibit distinct differences, as shown.

Survival in suspension culture

Esophageal and epidermal cells were tested for survival in suspension at early and later passages, when colony-forming efficiencies were low (several percent) and high (20 to 50%), respectively. As shown in Figure 5, colony-forming efficiencies decreased rapidly during the first day at a rate not substantially different from the 3- to 4-hr half-time reported for normal human epidermal cells (Rheinwald and Green, 1977; Rheinwald, 1979). Unlike the total loss of colony formation in the normal human cells (Rheinwald and Beckett, 1980), the rat cultures generally contained a small fraction that lost little germinative capacity between the second and third days of suspension. This fraction was normalized to the initial colony-forming efficiency of the cultures at zero time of suspension for comparative purposes. Thus, it became evident that the degree of survival, as normalized, was not substantially different in esophageal cells of the first and twentieth passages (1 to 2%). The absolute number of survivors capable of colony formation apparently increased simply as a result of a general increase in efficiency in the culture at large. In contrast, the degree of survival increased by an order of magnitude in the epidermal cells. As seen by comparison of the sixth and twentieth passages (Fig. 5), this effect reflected the very low initial survival (0.1%, normalized). Attempts to select esophageal cells from early passage for high colony-forming efficiency by suspension survival were unsuccessful, and progressive growth in suspension was not observed. Some clumping became apparent when the cells were held in suspension for up to 11 days, but colony-forming ability continued to decline slowly.

Twentieth passage esophageal cells were suspended for 3 days in methyl cellulose-stabilized medium. The surviving cells were expanded and given two more such cycles of suspension. The cell strain selected in this way was examined for suspension survival, as shown in Figure 5, and found to differ only slightly from the parent line, having a normalized survival of 6% versus 1 to 2%. Ionophore-inducible envelope formation was unaffected, since 91% of the selected cells were capable of forming envelopes. In a second type of experiment, it was found that passage of the parent esophageal cells three times at clonal density in the absence of feeder support also had little or no effect on survival in suspension. In all cases, colony morphologies and stratification in cultures

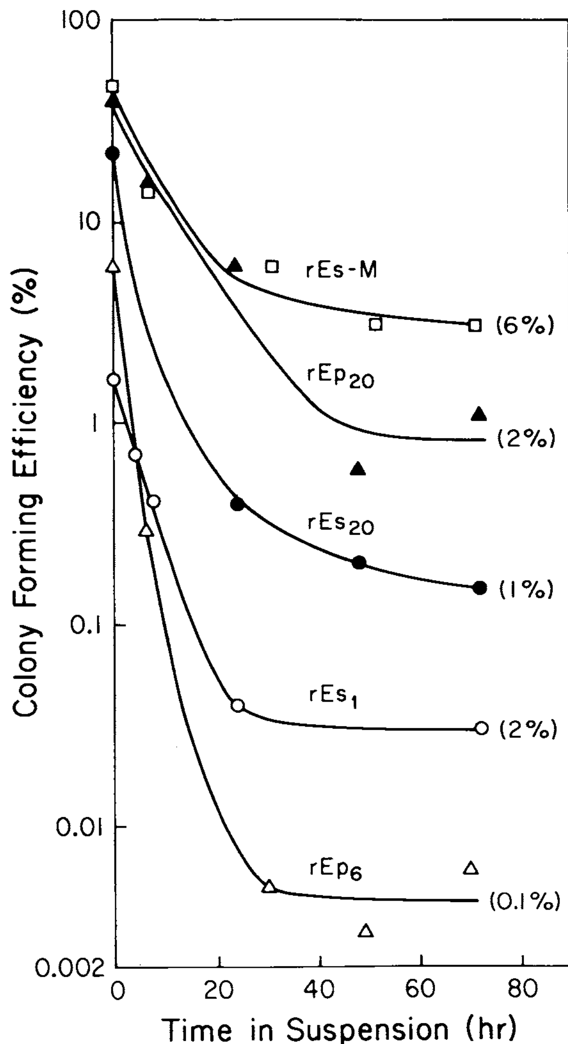

Fig. 5. Colony-forming efficiency of keratinocytes versus time suspended in semi-solid medium. The approximate percentages of 3-day survivors, divided by the initial colony-forming efficiencies, are given in parentheses for each experiment. rEs_1 and rEs_{20} represent rat esophageal cells of first and twentieth passage; rEp_6 and rEp_{20}, rat epidermal cells of sixth and twentieth passage; rEs-M, rat esophageal cells of twentieth passage previously given three cycles of alternate 3-day suspension in semi-solid medium and expansion of survivors in surface culture.

of the survivors were not substantially altered by the selection.

DISCUSSION

The present experiments show that rat esophageal and epidermal cells routinely become continuous lines upon serial pasasage. Until recently, such lines of rodent keratinocytes have been obtained only rarely without carcinogen treatment (Fusenig et al., 1978; Robine-Leon et al., 1981). A promising approach of inhibiting terminal differentiation by growth in low calcium medium (Hennings et al., 1980) has permitted generation of continuous lines of mouse epidermal cells with a range of properties (Yuspa et al., 1980), although a steady-state program of cell division and stratification with superficial differentiation is not observed. The rat keratinocytes of esophagus and epidermis, like those of lingual epithelium (Jepsen et al., 1980; Lillie et al., 1980; Birkedal-Hansen et al., 1981) clearly retain this coordinated program. Synthesis of keratin is in keeping with their stratified squamous character, although the differences in electrophoretic pattern seen in the natural epithelia were suppressed. This apparent convergence, perhaps due at least in part to vitamin A in the medium (Fuchs and Green, 1981), has also been observed in cultured rabbit keratinocytes (Doran et al., 1980). Since the latter were found capable of normal keratin expression upon subcutaneous transplantation in athymic mice, such cells provide opportunities for study of physiological influences modulating distinctive character.

The most striking difference between rat esophageal and epidermal keratinocytes concerns their regulation of cross-linked envelope formation. Epidermal cultures became highly competent to form ionophore-inducible envelopes only at confluence, whereas esophageal cells exhibited this property even in sparse culture. When the structural proteins participating in envelope assembly are identified and characterized, their differential expression and possible modulation by physiological agents will merit examination in the two cell types. Thus, levels of component(s) may be inversely coupled to growth in the epidermal (but not esophageal) cells, as is the case for human involucrin in malignant epidermal keratinocytes (Cline and Rice, 1983).

The two rat cell types also differed in rate of spontaneous envelope cross-linking in suspension, presumably due to altered rates at which they become permeable to calcium ion. This finding is consistent with recent reports that normal human epidermal and esophageal keratinocytes differ in rate of spontaneous envelope cross-linking in surface cultures as a function of retinyl acetate in the medium (Green and Watt, 1982), and that spontaneous cross-linking is suppressed by retinoic acid in suspended cells from guinea pig epidermis (Yaar et al., 1981). In both studies, the retinoids primarily affected cell permeability, since ionophore treatment produced envelope cross-linking to the same extent as in untreated cultures. In the present work, however, partial loss of competence was observed in the suspended cells, indicating some critical component (e.g., structural subunit, transglutaminase) is labile under these conditions. That the esophageal cells were reduced in competence by 50 to 70% in 1 day of suspension, but that envelopes did not appear in the remaining cells for several more days, suggests the putative labile component may be either not needed or not labile in the remaining one-third.

From evidence to date (Banks-Schlegel and Green, 1981; Green, 1980; Watt and Green, 1981, 1982), it is reasonable to conclude that normal human keratinocytes capable of envelope formation are outside the mitotic pool. By contrast, it was clear in the rat cells of late passage that envelope competence and germinative capacity were not mutually exclusive properties. For example, in sparse cultures of esophageal cells, at least one-third were capable of forming both colonies upon passage and envelopes upon ionophore treatment. In this respect, the rat cells resemble malignant human epidermal keratinocytes (Cline and Rice, 1983) but differ

from the latter in rapid loss of germinative capacity in suspension culture. This characteristic was relatively stable when allowance was made for the marked increase in colony-forming efficiency at later passages.

In the absence of obvious changes in envelope formation, keratin band pattern or colony morphology, the first indication of spontaneous cellular alteration upon serial passage was a dramatic 20- to 50-fold increase in colony-forming efficiency. Fibroblasts from rodents and other nonprimate species have long been observed to develop into continuous lines (McPherson, 1970), apparently through overgrowth by rare variants with unlimited life span (Todaro and Green, 1963). In the present work, it is plausible that the feeder layer provides a permissive environment for initial passages during which variants are generated. Clearly, any such variants were not selectable on the basis of enhanced survival in suspension culture. As shown as well with a keratinocyte line derived from a mouse teratoma (Morrissey and Green, 1978), this characteristic is not required for establishment of continuous lines. In any event, these observations have permitted subsequent generation of continuous cell lines from a variety of rat epithelia with 3T3 feeder layer support (Phillips and Rice, 1983).

ACKNOWLEDGMENTS

We thank Dr. P.R. Cline for suggestions on time course of envelope formation and Dr. J.G. Rheinwald for valuable discussions. This work was supported by U.S. Public Health Service Grants CA 27287 from the National Cancer Institute and AM 27130 from the National Institute of Arthritis, Metabolic, and Digestive Diseases.

LITERATURE CITED

Banks-Schlegel, S., and Green, H. (1981) Involucrin synthesis and tissue assembly by keratinocytes in natural and cultured human epithelia. J. Cell Biol., *90*:732–737.

Birkedal-Hansen, H., Hansen, I.L., Nellemann, K., and Westergard, J. (1981) Growth and differentiation of an established rat keratinocyte line in serial culture. In Vitro, *17*:553–562.

Cline, P.R., and Rice, R.H. (1983) Modulation of involucrin and envelope competence in human keratinoyctes by hydrocortisone, retinyl acetate, and growth arrest. Cancer Res., *43*:3203–3207.

Doran, T.I., Vidrich, A., and Sun, T.-T. (1980) Intrinsic and extrinsic regulation of the differentiation of skin, corneal and esophageal epithelial cells. Cell, *22*:17–25.

Fuchs, E., and Green, H. (1981) Regulation of terminal differentiation of cultured human keratinocytes by vitamin A. Cell, *25*:617–625.

Fusenig, N.E., Amer, S.M., Boukamp, P., and Worst, P.K.M. (1978) Characteristics of chemically transformed mouse epidermal cells in vitro and in vivo. Bull. Cancer, *65*:271–279.

Green, H. (1977) Terminal differentiation of cultured human epidermal cells. Cell, *11*:405–415.

Green, H. (1978) Cyclic AMP in relation to proliferation of the epidermal cell: A new view. Cell, *15*:801–811.

Green, H. (1980) The keratinocyte as differentiated cell type. Harvey Lect., *74*:101–139.

Green, H., and Watt, F.M. (1982) Regulation by vitamin A of envelope cross-linking in cultured keratinocytes derived from different human epithelia. Mol. Cell. Biol., *2*:1115–1117.

Hennings, H., Michaels, D., Cheng, C., Steinert, P., Holbrook, K., and Yuspa, S.H. (1980) Calcium regulation of growth and differentiation of mouse epidermal cells in culture. Cell, *19*:245–254.

Jepsen, A., MacCallum, D.K., and Lillie, J.H. (1980) Fine structure of subcultivated stratified squamous epithelium. Exp. Cell Res., *125*:141–152.

Laemmli, U.K (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, *227*:680–685.

Lillie, J.H., MacCallum, D.K., and Jepsen, A. (1980) Fine structure of subcultivated stratified squamous epithelium grown on collagen rafts. Exp. Cell Res., *125*:153–165.

McPherson, I. (1970) The characteristics of animal cells transformed in vitro. Adv. Cancer Res., *13*:169–215.

Moll, R., Franke, W.W., and Schiller, D.L. (1982) The catalog of human cytokeratins: Patterns of expression in normal epithelia, tumors and cultured cells. Cell, *31*:11–24.

Morrissey,, J.H., and Green, H. (1978) Differentiation-related death of an established keratinocyte line in suspension culture. J. Cell. Physiol., *97*:469–476.

Phillips, M.A. and Rice, R.H. (1983) Convergent differentiation in cultured rat cells from nonkeratinized epithelia: Keratinocyte character and intrinsic differences. J. Cell Biol., *97*:686–691.

Rheinwald, J.G. (1979) The role of terminal differentiation in the finite culture lifetime of the human epidermal keratinocyte. Int. Rev. Cytol. [Suppl.]*10*:25–33.

Rheinwald, J.G. (1980) Serial cultivation of normal human epidermal keratinocytes. Methods Cell Biol., *21*:229–254.

Rheinwald, J.G., and Green, H. (1975) Serial cultivation of strains of human epidermal keratinocytes: The formation of keratinizing colonies from single cells. Cell, *6*:331–344.

Rheinwald, J.G., and Green, H. (1977) Epidermal growth factor and the multiplication of cultured human epidermal keratinocytes. Nature, *265*:421–424.

Rheinwald, J.G., and Beckett, M.A. (1980) Defective terminal differentiation in culture as a consistent and selectable characteristic of malignant human keratinocytes. Cell, *22*:629–632.

Rice, R.H., and Green, H. (1979) Presence in human epidermal cells of a soluble protein precursor of the cross-linked envelope: Activation of the cross-linking by calcium ions. Cell, *18*:681–694.

Robine-Leon, S., Appay, M., Chevalier, G., and Zweibaum, A. (1981) Proliferation, differentiation and maturation of a mouse epidermal keratinocyte line. Exp. Cell Res., *133*:273–284.

Sun, T.-T., and Green, H. (1976) Differentiation of the epidermal keratinocyte in cell culture: Formation of the cornified envelope. Cell, *9*:512–521.

Sun, T.-T., and Green, H. (1978) Keratin filaments of cultured human epidermal cells. J. Biol. Chem., *253*:2053–2060.

Todaro, G.J., and Green, H. (1963) Quantitative studies of the growth of mouse embryo cells in culture and their development into established lines. J. Cell Biol., *17*:299–313.

Watt, F.M., and Green, H. (1981) Involucrin synthesis is correlated with cell size in human epidermal cultures. J. Cell Biol., *90*:738–742.

Watt, F.M., and Green, H. (1982) Stratification and terminal differentiation of cultured epidermal cells. Nature, *295*:434–436.

Wu, Y.-J., Parker, L.M., Binder, N.E., Beckett, M.A., Sinard, J.H., Griffiths, C.T., and Rheinwald, J.G. (1982) The mesothelial keratins: A new family of cytoskeletal proteins identified in cultured mesothelial cells and nonkeratinizing epithelia. Cell, *31*:693–703.

Yaar, M., Stanley, R., and Katz, S.I. (1981) Retinoic acid delays the terminal differentiation of keratinocytes in suspension culture. J. Invest. Dermatol., *76*:363–366.

Yuspa, S.H., Hawley-Nelson, P., Koehler, B., and Stanley, J.R. (1980) A survey of transformation markers in differentiating epidermal cell lines in culture. Cancer Res., 4694–4703.