

## Altered pattern of growth and differentiation in human keratinocytes infected by simian virus 40

(viral transformation/epithelium)

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**ABSTRACT** Human epidermal keratinocytes were infected by simian virus 40 *in vitro*. The structure of the developing keratinocyte colony reflects the spatial separation of cell division and keratinization in intact skin; thymidine-incorporating cells were primarily localized at the colony periphery whereas nondividing, histologically differentiated cells accumulated in the interior. Viral infection produced a dramatic increase in the size of the proliferative population as, simultaneously, differentiation was reduced in the colony interior. These changes were manifest when simian virus 40 T-antigen synthesis was detectable in only a small percentage of the cells; differentiation became increasingly density dependent as the percentage of T-antigen-positive cells rose over serial passage. The disruption of the normal pattern of growth/differentiation localization coincided with a loss of dependence on serum for growth, but preceded the appearance of other virus-induced properties associated with transformation; i.e., the ability to form colonies in soft agar and independence of growth from fibroblasts.

Epidermis is a tissue in which differentiation is a process confined to a single cell type and defined geographically by the position of each cell in the tissue structure. These characteristics offer considerable advantages for the study of differentiation in comparison to other systems in which the developmental sequence is not directly related to tissue architecture or is dependent upon the influence of intervening effectors. In the past, research in this system has been impeded by the lack of adequate culture conditions for sustaining epidermal cell growth *in vitro*. Recently, however, a culture method has been developed that permits long-term serial cultivation of epidermal cells derived from human skin and that supports the growth of colonies probably arising from single cells (1). In this system the developmental process of epidermal architecture is maintained in terms of a geographical relationship between the replicating stem population and the terminally differentiating cells (1, 2).

Using this system we have studied changes in the normal patterns of growth and differentiation resulting from infection of human epidermal cells by simian virus 40 (SV40). Our findings indicate that these processes are highly sensitive to viral infection and that modification of the normal pattern of growth and differentiation in the keratinocyte colonies are detectable prior to other parameters of cell transformation.

### METHODS AND MATERIALS

**Cell Culture and Virus Infection.** Human epidermal keratinocyte cultures were prepared from tissue specimens as described by Rheinwald and Green (1). Briefly, freshly excised skin specimens of neonatal foreskin were washed in culture medium, minced with scissors, and trypsinized (0.25% trypsin, GIBCO) with rapid stirring for 45 min at room temperature.

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Cells in the resulting suspension were pelleted by centrifugation resuspended in fresh culture medium, and plated with feeder cells that had been prepared by exposing a suspension of newly harvested 3T3 cells to a  $^{137}\text{Cs}$  source in a rotary gamma irradiator (4800 rads, 48.00 J/kg). The growth medium used was Dulbecco's modified minimal essential medium (GIBCO), supplemented with 10% (vol/vol) fetal calf serum (Flow Laboratories, Rockville, MD) and 0.4  $\mu\text{g}$  of hydrocortisone per ml. For passage, cultures were trypsinized when confluent and replated in the presence of irradiated feeder layers.

For viral infection, trypsinized epidermal cells (approximately  $10^5$ ) were suspended in culture medium containing  $1.8 \times 10^7$  plaque forming units of SV40 (strain Rh 911) per ml and incubated at 37°C as a loosely packed suspension for 4–6 hr in an atmosphere of 5%  $\text{CO}_2$  prior to plating with the feeder cells. Preliminary experiments have shown that infection of freshly trypsinized cells in suspension is more efficient than when virus is added to already formed monolayers.

**Immunofluorescent Staining for SV40 T and V Antigens.** Cells were stained for SV40 T [the product of the early SV40 gene(s)] and V (viral capsid protein) antigens by the indirect immunofluorescence technique (3) by using antiserum against SV40 T antigen from SV40 tumor-bearing hamsters or rabbit antiserum against SV40 and fluorescein isothiocyanate-conjugated rabbit anti-hamster or goat anti-rabbit serum (Antibodies Incorporated, Davis, CA).

**Autoradiography.** Keratinocyte cultures on coverslips were incubated in culture medium containing 2  $\mu\text{Ci}$  of [ $^3\text{H}$ ]dThd per ml (20–40 Ci/mol) or 4  $\mu\text{Ci}$  of [ $^{14}\text{C}$ ]dThd per ml (50 Ci/mol) (both from New England Nuclear) for 2 hr. For standard autoradiography, coverslip cultures were washed in phosphate-buffered saline and fixed in Carnoy's solution. When immunofluorescence was to be combined with autoradiography, coverslips were fixed in cold acetone/methanol (70:30, vol/vol) and stained for T antigen prior to autoradiography. In some experiments, microscopic fields were projected onto paper screens with a Leitz microprojector, the epithelial colony perimeters were traced, and the location of labeled and unlabeled nuclei was recorded. By using a compass, the tracing of each colony was further subdivided into concentric regions whose borders maintained a constant distance from the colony edge as indicated.

**Staining of Cells for Keratin Proteins.** Formalin-fixed cells were stained for keratin proteins by Ayoub and Shklar's modification of Mallory's method (4) which uses acid fuchsin followed by an orange G/aniline blue counterstain. By this procedure prekeratin is stained orange and keratin is stained red, while fibroblasts and undifferentiated keratinocytes are stained blue. In sections of human skin, only the upper keratinizing layers and the keratin found in hair follicles are stained red or orange. Confluent cultures of African green monkey kidney

Abbreviation: SV40, simian virus 40.

cells or of HeLa cells, which contain noncornified epithelial cells, are stained blue.

**Growth in Agar.** Keratinocytes were harvested and suspended in soft Noble agar (final concentration 0.5%) to test for the anchorage dependence of their growth capacity (5).

## RESULTS

**Characteristics of Normal and SV40-Infected Epidermal Cultures.** Within 1–2 weeks after seeding, cultures of epidermal keratinocytes consist of small circular colonies composed of tightly packed polygonal cells with a characteristically epithelial appearance. Fig. 1A illustrates that, typically within each emerging colony, two geographical regions can be distinguished: an undifferentiated (blue-staining) peripheral monolayer and a keratinized (orange- and red-staining) multilayered interior. Subsequent growth of each colony occurs by lateral expansion of the colony periphery and by progressive multilayering of the newly generated interior region(s), a process resulting in larger colonies containing proportionately larger regions of keratinizing cells. As the epithelial colonies expand, the fibroblast layer is progressively compressed into thick strands and is progressively displaced as the cultures are maintained. This pattern of growth and expansion is maintained through several passages, when the cultures are trypsinized and replated with fresh 3T3-feeder layers. However, after four to six passages, the epithelial colonies grow only to a smaller size and the interior of the colonies are also much thicker with a predominance of keratinized areas.

In the infected cultures, only a few cells in each of the several epithelial colonies became positive for SV40 T antigen in the initial days after infection. As the cultures were passaged, the percentage of T-antigen-immunofluorescent cells progressively

Table 1. Properties at various passages of SV40-infected keratinocytes

Passage	% T-antigen-positive		% V-antigen-positive cells	Growth of uninfected cultures*
	Colonies	Cells		
1	30.8	2.7	0.47	+++
2	53.2	5.4	0.34	+++
3	87.5	16.7	0.11	++
4	100	47.9	0.44	++
5	100	54.8	0.33	–
10	100	79.7	0.70	–
15	100	85.5	0	–

\* Pluses indicate the extent of replication of keratinocytes in the colonies.

increased, as has been often described when only a minority of the cell population is initially infected (3). A representative example of this type of progression is illustrated in Table 1. At 2–3 weeks after infection only about 30% of the colonies had T-antigen-positive cells; within the positive colonies, the percentage of positive cells varied by a small range and the positive cells were randomly distributed within each colony. The irradiated 3T3 cells showed a high proportion of T-antigen-positive cells, whereas the proportion of positive human fibroblasts were very low. After the initial passage, the number of colonies with positive cells increased very rapidly, and by the fourth passage (Table 1) each colony had some positive cells. This reflects the cell reassortment that occurs during trypsinization and replating and the fact that T-antigen-positive cells plate at a higher efficiency (unpublished observation). By the 15th passage, about 85% of the cells were T antigen positive. The T-antigen-positive

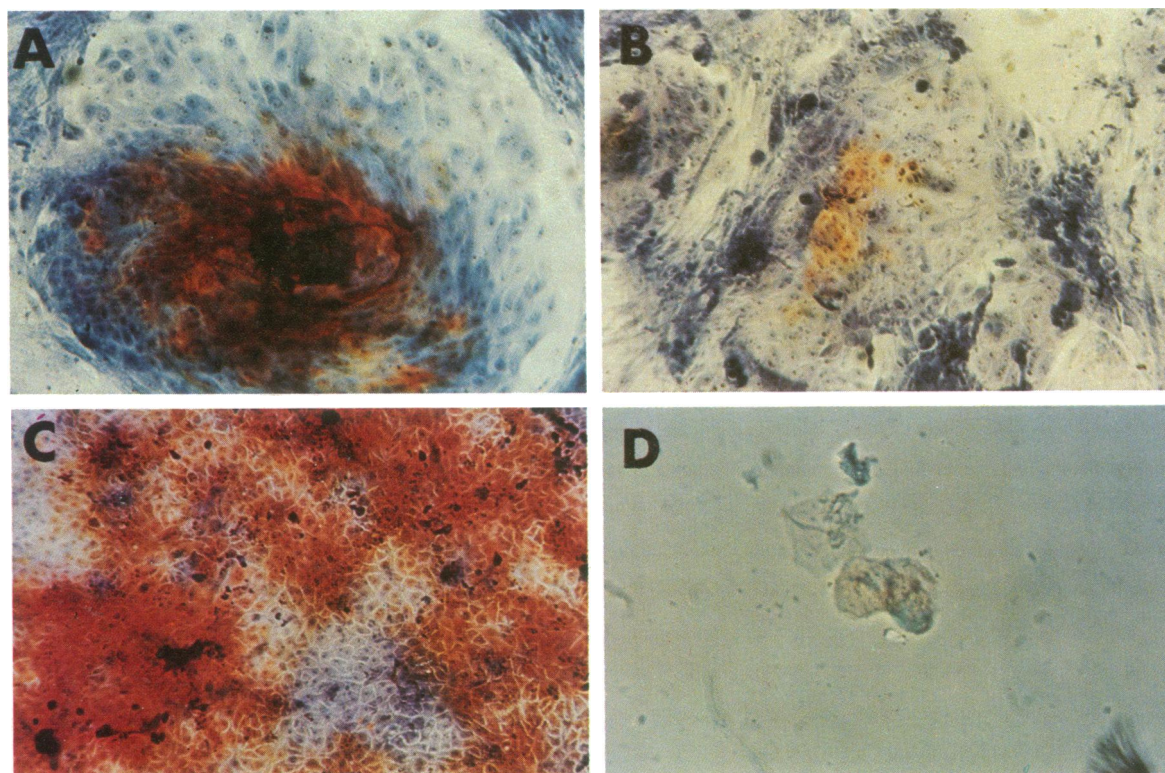


FIG. 1. Normal and SV40-infected stain C epidermal keratinocytes. ( $\times 80$ .) (A) Normal keratinocyte colony from a second-passage culture fixed and stained 14 days after seeding. (Ayoub-Shklar stain.) (B) SV40-infected keratinocyte colony from a fourth-passage culture 14 days after seeding. (Ayoub-Shklar stain.) (C) SV40-infected keratinocytes from a confluent monolayer culture, seeded in the absence of a fibroblast feeder layer. (Ayoub-Shklar stain.) (D) Cluster of enucleate squamous-like cells shed from a culture of SV40-infected keratinocytes (sixth passage). (Papanicolaou stain.)

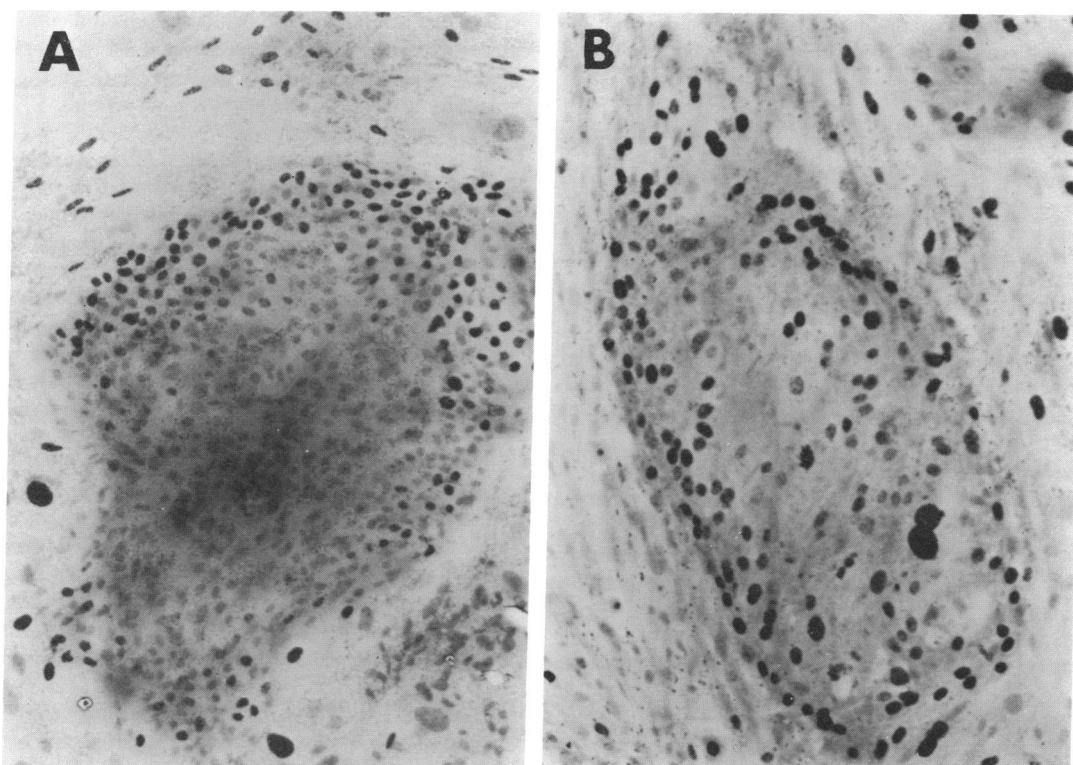


FIG. 2. Autoradiography of normal and SV40-infected keratinocytes. Keratinocyte cultures were exposed for 2 hr to [ $^3\text{H}$ ]dThd ( $2 \mu\text{Ci/ml}$ ). (A) Colony from a normal primary culture. ( $\times 100$ .) (B) Colony from an SV40-infected fourth-passage culture. ( $\times 100$ .)

3T3 cells and human fibroblasts eventually disappeared after the early passages.

Cells producing V antigen at any time represented a very low percentage, and their frequency had no relationship to the proportion of T-antigen-positive cells. In the case illustrated in Table 1, they virtually disappeared by the 15th passage.

The morphological appearance of the epithelial colonies in the infected cultures, even when 50–70% of the cells were T antigen positive, was quite similar to the controls. However, the infected colonies appeared flatter and increased in size more rapidly without reaching high cellular density (Figs. 1B and 2B); they did not show cornified multistratified layers, but shed a large number of cells in the culture medium, many of which appeared to be fully cornified enveloped squames (Fig. 1D) similar to those described in noninfected cultures (2, 6). Also, the epithelial cells appeared to be more loosely packed, with a considerable degree of anisocytosis.

Another important feature of infected cultures is illustrated in Table 1. The epithelial cells in this experiment could not be effectively transferred beyond the fifth passage, indicating that the growth capacity to form colonies was practically exhausted. The infected cultures, however, continued to grow vigorously even when a portion of the cell population was still T antigen negative.

**Labeling Pattern in Normal and SV40-Infected Epidermal Cells.** [ $^3\text{H}$ ]dThd incorporation in epidermal cell colonies is confined mainly to cells located in the colony periphery. The autoradiographic analysis of the uninfected colonies in Figs. 2 and 3 show that the labeling of cells is maximal within the first 20–60  $\mu\text{m}$  of the colony perimeter and declines sharply over the next 60–120  $\mu\text{m}$ . This labeling pattern is remarkably constant among different cell strains and at different passage levels (Fig. 3A), and by and large it is independent of the colony size. In the set of data in Fig. 3A, 31% of all the labeled cells were located within the first peripheral 20  $\mu\text{m}$  while only 4% were

found at 100 and 120  $\mu\text{m}$  from the edge (the diameters of the colonies in this estimation were between 250 and 400  $\mu\text{m}$ ). Similar results were obtained when [ $^{14}\text{C}$ ]dThd was used, the only difference being that a slightly higher percentage of labeled cells was observed if the measured areas contained the thicker inner regions of the colonies ( $>100 \mu\text{m}$  from the margin).

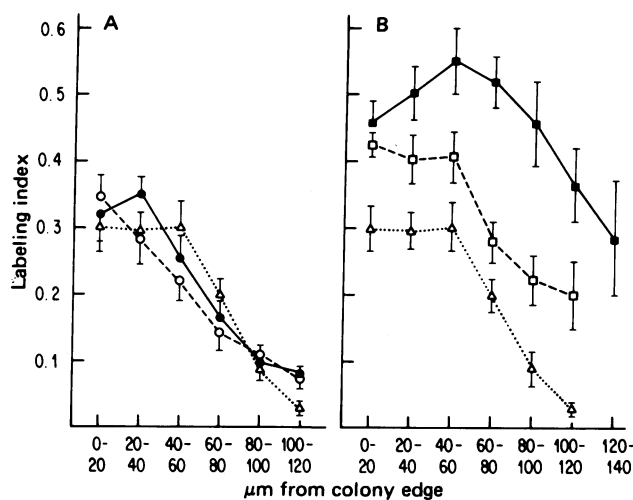


FIG. 3. Geographic distribution of labeled nuclei in normal and SV40-infected keratinocyte colonies after exposure to [ $^3\text{H}$ ]dThd ( $2 \mu\text{Ci/ml}$ ) for 2 hr. Values represent the percentage of labeled nuclei in 20- $\mu\text{m}$  colony segments averaged over 10–12 colonies. Bars represent SEM. (A) Distribution of labeled nuclei in normal keratinocyte colonies from different strains at various passages:  $\Delta$  - - -  $\Delta$ , strain B;  $\circ$  - - -  $\circ$ , strain C;  $\bullet$  - -  $\bullet$ , strain D. (B) Distribution of labeled nuclei in normal and SV40-infected strain B keratinocyte colonies.  $\Delta$  - - -  $\Delta$ , Uninfected cells;  $\square$  - - -  $\square$ , 17% T antigen positive;  $\blacksquare$  - -  $\blacksquare$ , 71% T antigen positive.

Table 2. [<sup>3</sup>H]dThd incorporation into T-antigen-positive and T-antigen-negative SV40-infected cells

Exp.	% T-antigen-positive cells	% T-antigen-negative cells	% all cells
I	56.9	28.8	46.3
II	54.0	37.9	47.4

Autoradiography of SV40-infected keratinocyte colonies labeled for 2 hr with [<sup>3</sup>H]dThd (2  $\mu$ Ci/ml) was performed after cells were stained for T antigen.

In contrast, the labeling pattern of SV40-infected cultures differed from the comparable control cultures in two important aspects (Figs. 2 and 3): (i) a higher labeling index was observed in all regions of the colonies and (ii) labeled cells were present at a higher frequency in the internal regions of the colonies. In the infected cultures, the labeling gradient from the periphery to the center of each colony was still maintained (Fig. 3B). Changes of the labeling pattern were evident even when only a fraction of the population was T antigen positive; for example (Fig. 3B), the circular section from 100 to 120  $\mu$ m in the infected cultures with an average of 17% of T-antigen-positive cells had the same labeling index as the control cultures at 60–80  $\mu$ m (Fig. 3A). As the percentage of immunofluorescent cells increased, the deviation from the normal labeling pattern was higher (Fig. 3B). When the same cultures were concomitantly scored for T-antigen immunofluorescence and for [<sup>3</sup>H]dThd incorporation (2-hr pulse), it was evident that the labeling index was higher for the T-antigen-positive cells than for the negative cells present within the same colonies (Table 2), consistent with the mitogenic effect of the SV40 T antigen (7). It was also significant that in the two experiments reported in Table 2, the labeling index of the T-antigen-negative cells was higher than would be expected in similar regions of uninfected cultures.

One of the best documented differences between normal and transformed cells is the capacity of the latter to replicate in medium containing low amounts of serum (8, 9). This property was tested by maintaining control and infected cultures at a low serum concentration (0.15% fetal calf serum) and then exposing them to a 2-hr pulse of [<sup>3</sup>H]dThd at daily intervals. As illustrated in Fig. 4, in the control cultures the labeling index rapidly de-

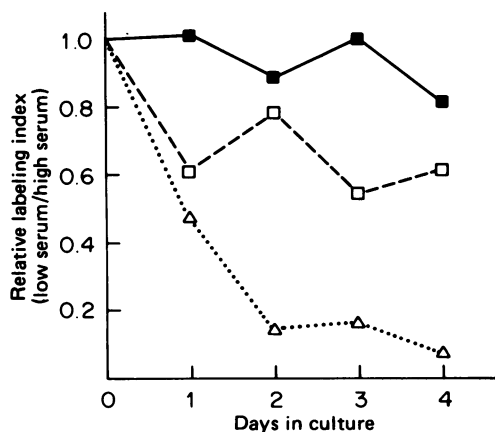


FIG. 4. Serum dependence of [<sup>3</sup>H]dThd incorporation in normal and SV40-infected strain B keratinocyte colonies. Cultures were grown in culture medium containing either high (10%) or low (0.15%) serum concentrations. At the indicated times, cultures were incubated for 2 hr in the appropriate culture medium containing [<sup>3</sup>H]dThd (2  $\mu$ Ci/ml). Average labeling indices were determined from count of autoradiograms of 35 colonies.  $\Delta \cdots \Delta$ , Uninfected;  $\square - - \square$ , 17% T antigen positive;  $\blacksquare - \blacksquare$ , 71% T antigen positive.

clined to a low level by 48 hr. In the infected cultures, however, almost no decline was observed over the 4-day period when 71% of the cells were T antigen positive (sixth passage), and there was only a minimal decline even when only 17% of the cells were T antigen positive.

**Other Properties of Infected/Transformed Epithelial Cultures.** The capacity of keratinocytes to replicate and differentiate *in vitro* is dependent upon the presence of intermixed fibroblast feeder cells (1). Growth of infected keratinocytes, however, became independent of fibroblasts between the fifth and seventh passage (about 70% T-antigen-positive cells). Thus, the epithelial cells could be transferred without the addition of 3T3 cells although an initial high density of inoculum was needed and the growth rate was considerably reduced with respect to parallel cultures still passaged in the presence of 3T3 cells. Eventually, upon further passage, cultures of pure epithelial cells originating from single cells could be obtained. In the pure epithelial cells, differentiation still occurred, as demonstrated by extensive staining for keratin proteins in areas of high cell density (Fig. 1C); however, multilayered formations, which are numerous in the noninfected cultures, were scarce in these cultures while shedding of cornified cells and squames in the supernatant was abundant. These squames were resistant to sodium dodecyl sulfate in the presence of 2-mercaptoethanol, demonstrating disulfide-bonded keratins (6).

Upon examination under the electron microscope, the infected keratinocytes maintained the properties of the epithelial cells with a high number of desmosomal complexes and thick bundles of tonofilaments. In addition, transformed cells had many more microvilli than the corresponding normal keratinocytes.

Normal keratinocytes do not produce colonies in semisolid medium (6). In contrast, when infected cells from three different strains were tested at a time when more than 50% of the cells were T antigen positive, colonies formed in soft agar in all cases with a colony-forming efficiency that varied from about 1 to  $6 \times 10^{-4}$ . The colonies were spherical and uniform in appearance and the margins of the tightly packed cells were indistinct. When removed from the agar, colonies readily attached to plastic surfaces, but outgrowth was very slow whether they were plated in the presence or absence of the 3T3 feeder layer and consisted of T-antigen-positive cells.

## DISCUSSION

In this report we have characterized the effect of the oncogenic virus SV40 on the growth and differentiation of human epidermal cells *in vitro*. Because of the unique properties of this system, it has been possible to delineate a new significant pattern of altered growth regulation induced by this virus. In normal epidermal cultures, the differentiation scheme can be mapped with respect to the geography of the developing colonies: the colony periphery (undifferentiated, dividing cells) and the colony interior (differentiated, nondividing cells) as geographical regions representing the extremes of the differentiation process. The localization of proliferating cells is quite striking. Reduction in the percentage of cells engaged in DNA synthesis is detectable within a few cell diameters of the colony perimeter and declines continuously toward the center of the colonies. The reduction in the proportion of labeled cells in the more differentiated regions suggests that the epidermal cell cycle is continuously lengthened prior to terminal differentiation and that noncycling cells deep within the colony interior are the immediate precursors of the fully keratinized squames that comprise the central superficial layers. The basic features of this pattern of growth and differentiation are not changed by viral infection, but there is a virus-related shift in the spatial

distribution of cells in that the proliferative populations occupy larger portions of the periphery while differentiation simultaneously becomes more restricted within the interior as the proportion of SV40 T-antigen-positive cells increases over serial passage. These changes occur at the time when the proportion of T-antigen-positive cells represent a minority of the cell population (for example, 17% in Fig. 3) and long before the appearance of other transformed properties; e.g., growth independence from substrate attachment and from fibroblasts. Since growth and differentiation of epidermal cells have been postulated to be controlled by chemical gradients based upon direct (i.e., junctional) cell-to-cell communication (10, 11), it is possible that through desmosomal complexes the mitogenic stimulation of the T antigen diffuses to neighboring noninfected cells.

The disruption of regional specificity described here parallels the findings in other studies in which [<sup>3</sup>H]dThd-incorporating cells were observed in normally nondividing differentiated strata in epithelial tumors of viral origin (12).

The results reported here also emphasize the difference and the similarity between the transformed phenotypes of fibroblasts and epithelial cells. The altered pattern of [<sup>3</sup>H]dThd incorporation occurs during the early stage of infection when there is no obvious morphological difference between normal and infected cultures, a considerable difference from the transformed foci of fibroblastic cells. However, the transformed epithelial cells show a reduced dependence of serum for growth; the capacity to multiply in the absence of fibroblast feeder cells and to form colonies in semisolid agar, albeit to a low incidence; and a great increase in life span *in vitro*.

The transformed cultures maintain the capability to differentiate and to produce cornified cell squames, although mul-

tilayer strata are greatly reduced; extensive keratinization occurs only when high cell density is reached. The capacity to differentiate is maintained even though the transformed cells are grown without fibroblasts, a fact that is consistent with the evidence obtained by Green (6), that there is no direct influence of fibroblasts on the process of differentiation *per se*.

Since the SV40-transformed cells can be cloned and most of the clones eventually produce keratin (unpublished data), genetic analysis of epithelial differentiation becomes possible.

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