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Authors

Phillips, MA
Rice, RH

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Convergent Differentiation in Cultured Rat Cells from Nonkeratinized Epithelia: Keratinocyte Character and Intrinsic Differences

MARJORIE A. PHILLIPS and ROBERT H. RICE

Charles A. Dana Laboratory of Toxicology, Harvard School of Public Health, Boston, Massachusetts 02115

ABSTRACT Epithelial cells derived from a variety of glandular and other nonkeratinized rat tissues (pituitary, thyroid, bladder, endometrium, trachea, seminal vesicle, prostate, and mammary epithelium) were serially cultivated using a feeder layer of lethally irradiated 3T3 cells. The epithelial cells grew as progressively expanding colonies, in some cases stratified, and were shown to form cornified envelopes upon ionophore-induced activation of cross-linking. Cultures derived from each tissue were distinguishable from the others by characteristic cellular appearance and colony morphology. Those examined in greater detail could be distinguished biochemically in three ways. (a) A majority of cells in sparse cultures of bladder, tracheal, endometrial, and vaginal epithelial cells were capable of envelope formation, whereas those from pituitary, thyroid, seminal vesicle, and mammary epithelia did not attain maximal envelope forming ability until after confluence. (b) Bladder, thyroid, and pituitary cells exhibited different electrophoretic profiles of keratins, which accounted for 20–50% of the cellular protein. (c) Bladder cells were distinguished from thyroid and pituitary cells by a greater suppression of envelope-forming ability by vitamin A. These observations showed that cells from many epithelia have the potential to express properties of keratinocytes in culture while maintaining morphological and physiological differences. Serial passage of these cells generated continuous lines.

Recent advances in technique now permit serial cultivation with feeder layer support of keratinocytes from epidermis and other stratified squamous epithelia (26, 27). In culture, these cells display the two major structural features that distinguish them from other epithelial cells, the cross-linked (or “cornified”) envelope and a high keratin content (8). The envelope, a protein structure stabilized by transglutaminase cross-linking (29), is found immediately beneath the plasma membrane (6). Only a small fraction of cultured epidermal keratinocytes possesses envelopes (34), but a large majority will synthesize them rapidly upon treatment with calcium ionophores, thereby activating the cellular transglutaminase (30). Keratins, a family of insoluble intermediate filament proteins, are found in virtually all epithelial cells, but in keratinocytes they constitute an exceptionally high percentage (30% or more) of cell protein (35). Although keratinocytes cultivated from various stratified squamous epithelia show considerable similarity in their differentiation programs, they can be distinguished under certain conditions by their gel electrophoretic patterns of

keratins (4) and by their sensitivity to vitamin A suppression of spontaneous envelope cross-linking (10).

In contrast to the keratinocyte, functional cells of other epithelia have proven difficult to propagate in culture (39). Usually, this difficulty arises from loss of differentiated character or from fibroblast overgrowth. Functional cell lines derived from tumors have proven valuable but can be difficult to establish and hence are uncommon. A few differentiated cell lines have been established from normal rat tissue: endometrial cells expressing estrogen receptor activity (33); fetal pituitary clones secreting prolactin, growth hormone, and adrenocorticotrophic hormone (14); and thyrotropin-responsive cells secreting thyroglobulin (1). These findings suggest that retention of differentiated character in a given cell type requires a specially tailored environment. The present results show that cell lines were indeed serially cultivable, with feeder layer support, from a variety of glandular and other nonkeratinized rat epithelia. These lines exhibited morphological and biochemical differences, but shared expression of keratin-

ocyte character. Studying this shift in program, resembling squamous metaplasia *in vivo*, may help elucidate control mechanisms regulating the cellular repertoire of differentiated function.

MATERIALS AND METHODS

Cell Culture: The epithelial cells from Sprague Dawley rats were cultivated with feeder layers of lethally irradiated mouse 3T3 cells according to standard procedures (26). For some experiments, primary cultures of pituitary were initiated as disaggregated cell suspensions prepared by treatment with collagenase (Worthington type I [Worthington Biochemical Corp., Freehold, N.J.], 500 U/ml) and DNase I (Sigma type III [Sigma Chemical Corp., St. Louis, MO], 5 µg/ml) for 1–2 h at room temperature with occasional pipetting. After several washes in medium, the cells were inoculated at $\sim 10^5$ cells/60-mm dish with $\sim 4 \times 10^5$ 3T3 feeder cells. Explants of minced pituitary or other tissues were also cultivated with feeder layers, since epithelial outgrowth was considerably stimulated. Mammary gland tissue was taken on approximately the seventeenth day of pregnancy. All cultures were passaged with new feeder layers after removal of contaminating tissue fibroblasts and remaining 3T3 cells with isotonic 0.02% disodium EDTA (34) and subsequent trypsinization. In some cases, repeated removal of fibroblasts with EDTA and replacement of the feeder layer were necessary during the first several passages. For calculation of colony-forming efficiencies, macroscopic colonies of at least 50 cells were scored in cultures stained with rhodanile blue (27).

Cells were grown in Dulbecco-Vogt Eagle's medium supplemented with fetal bovine serum (20%), hydrocortisone (0.4 µg/ml), epidermal growth factor (10 ng/ml), and cholera toxin (9 ng/ml). For observation of vitamin A effects, serum supplementation was 10% and the medium was changed at 2-d intervals. The retinyl acetate concentration at each medium change was adjusted by fresh dilution from a 4 mg/ml stock solution in dimethyl sulfoxide stored at -70°C under N_2 gas between uses.

Measurement of Cross-linked Envelopes: Freed of 3T3 and tissue fibroblasts by EDTA treatment, the epithelial cells were trypsinized, counted, and suspended in serum-free medium containing the ionophore X537A (50 µg/ml), which activates transglutaminase-mediated cross-linking (30). After 2 h at 37°C , the samples were adjusted to 2% in SDS and 20 mM in dithioerythritol. Detergent-resistant envelopes were counted under phase contrast optics when the keratins and other cytoplasmic proteins had dissolved (after ~ 20 min at room temperature). Values from duplicate samples differed by 10% or less.

Analysis of Keratins: After removal of feeders and any tissue fibroblasts with EDTA, cultures were scraped into ~ 1 ml of a 10 mM Tris-HCl (pH 7.4)-1 mM EDTA buffer. The cells were extracted twice with 10 mM Tris-1 mM EDTA-1% Triton X-100 buffer and twice with this buffer adjusted to 0.45 M in NaCl. The insoluble keratins, recovered by centrifugation at 8,000 *g* for 3 min between extractions, were dissolved by boiling in 10 mM Tris-HCl (pH 6.8)-2% SDS-1 mM dithioerythritol and submitted to electrophoresis in 8% polyacrylamide slab gels (17). The separated proteins were then transferred electrophoretically to nitrocellulose paper and stained either with Amido black or by the immunoperoxidase reaction (37) using 4-chloro-1-naphthol (Bio-Rad Laboratories, Richmond, CA) as electron donor. Rabbit antiserum raised against total keratin of human stratum corneum was generously provided by Dr. J. G. Rheinwald (Harvard University). When preimmune serum was substituted for antiserum, no staining was observed. Estimates of protein concentrations in Triton-soluble and -insoluble fractions of the crude extracts were obtained as described by Lowry et al. (20) in 0.05% SDS and corrected for the small contribution of dithioerythritol. Duplicate values within an experiment differed by $<10\%$.

RESULTS

In initial experiments, disaggregated epithelial cells from rat pituitary grew little if at all in primary cultures prepared by traditional techniques and did not passage successfully. Tissue fibroblasts proliferated rapidly and appeared to be the only cell type surviving passage. In contrast, when the disaggregated cells were co-cultivated with a feeder layer of lethally irradiated 3T3, tightly packed, progressively expanding colonies of typical epithelial morphology were observed (Fig. 1*a*). Both the rate of colony expansion and efficiency of colony formation upon subsequent passage were considerably increased by supplementation of the medium with epidermal growth factor

and cholera toxin at concentrations effective in human epidermal cells (7, 28). Hydrocortisone, slightly beneficial in some experiments, was also included. The appearance of these epithelial colonies was not absolutely dependent upon 3T3 cells, since they were observed in primary cultures without the feeder layer; in the presence of epidermal growth factor and cholera toxin, the abundant tissue fibroblasts supported establishment of epithelial colonies, but rapid proliferation of the fibroblasts subsequently inhibited epithelial expansion and prevented subculture.

Derivation of Continuous Lines

In primary culture under optimal growth conditions, the disaggregated pituitary cells had colony-forming efficiencies of 0.1–0.5%. There was no obvious difference between male and female tissue nor between separated anterior and intermediate lobes. When epithelial outgrowths from pituitary explants were passaged, they gave rise to colonies of the same appearance as obtained from enzymatically disaggregated tissue. Epithelial outgrowths from rat thyroid and bladder explants were also found to passage readily with 3T3 feeder layer support (Fig. 1, *b* and *c*). Upon serial subcultivation the colony-forming efficiencies rose progressively 50–100-fold and stabilized in the range of 20–40% within 10 passages. The cells have been in culture over 150 generations with no indication of senescence and must be considered continuous lines. Cultures of all three epithelial origins were observed to form the same swirling patterns as those found in human epidermal cells (9).

The different epithelial cultures were easily distinguishable microscopically despite some variation in morphology among individual colonies in a given dish. The pituitary cells were generally smaller than the others and the bladder cells had considerably more prominent nuclei. Close inspection under phase-contrast optics revealed large flat squames on the surface of thyroid and pituitary, but not bladder, colonies. The thyroid colonies often contained small areas that appeared at low magnification to be devoid of cells (Fig. 1*b*) but in many cases were covered with squames.

Expression of Envelopes and Keratins and Effects of Retinyl Acetate

The microscopic observation of superficial squames in pituitary and thyroid colonies indicated some stratification. Thus, the possibility that these cells exhibited keratinocyte character was explored by measuring their competence in cross-linked envelope formation. In confluent cultures treated with the ionophore X537A, which activates cellular transglutaminase by permitting an influx of calcium ions (30), a majority of each cell type formed envelopes, but the degree of competence in sparse culture (<50 cells/colony) depended upon the cell type (Fig. 2). The bladder cells were highly competent in sparse culture, whereas pituitary and thyroid cells increased in competence about fourfold from the earliest time the cells were readily testable at day 4 until confluence 4–6 d later.

The pituitary cells did not require extended passage to develop envelope competence since primary cultures initiated from collagenase-digested tissue exhibited considerable envelope-forming ability (Fig. 2*a*). Moreover, the early outgrowths of 4-d pituitary (as well as thyroid and bladder) explants, treated in the culture dish with ionophore and then detergent

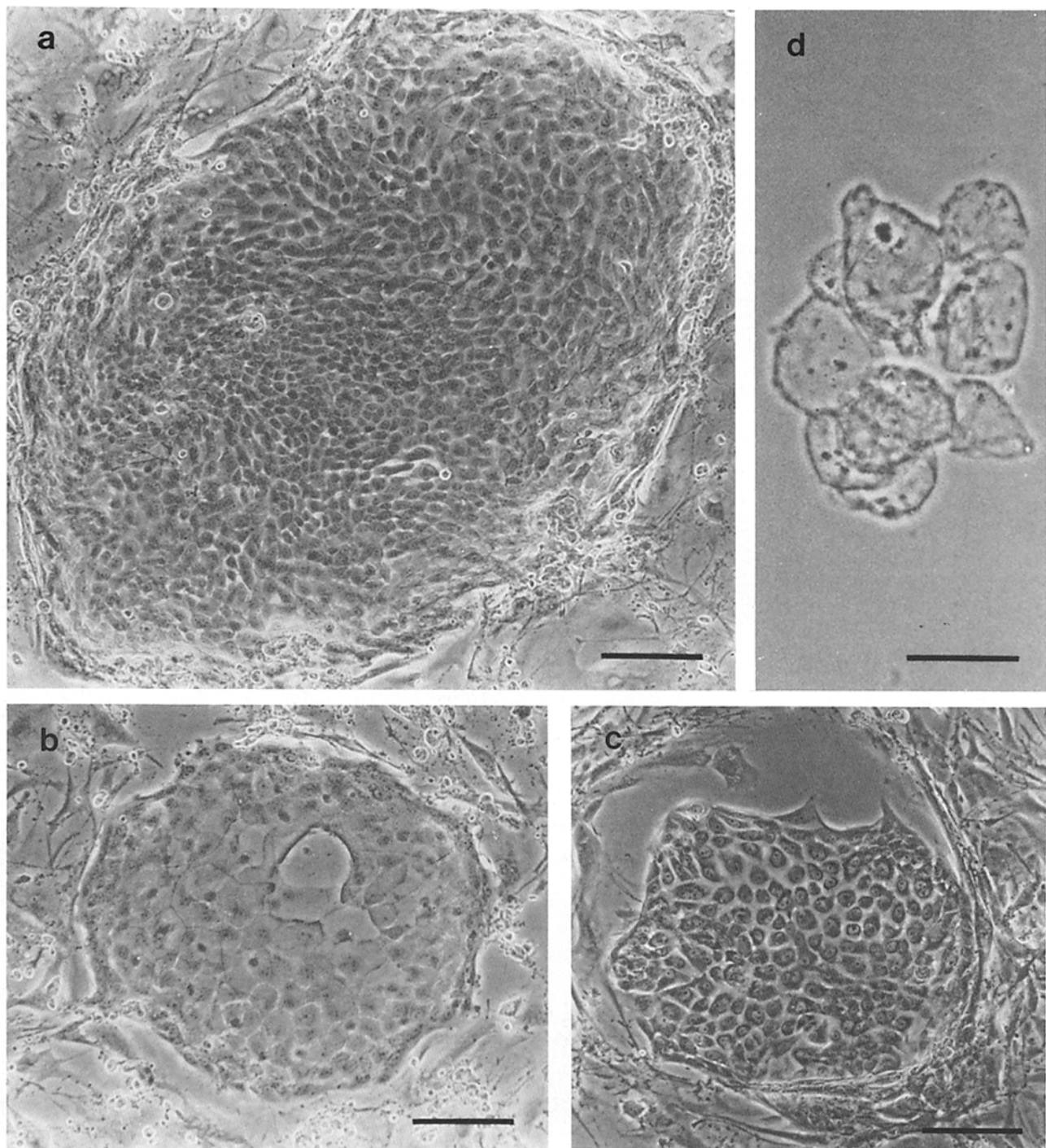


FIGURE 1 Epithelial colonies from (a) anterior pituitary, fifth passage; (b) thyroid, third passage; and (c) bladder, third passage; (d) cross-linked envelopes induced with ionophore in secondary surface cultures of prostate epithelium, visualized after treatment with detergent and reducing agent. Bars, 50 μm (a-c), 15 μm (d).

and reducing agent, formed obvious envelopes all around the tissue periphery. In contrast, <0.5% of the cells isolated directly from fresh pituitary tissue either possessed envelopes or were capable of forming them upon ionophore treatment.

Retinyl acetate and retinoic acid have recently been found to suppress spontaneous envelope cross-linking in cultured human epithelial keratinocytes (10) and suspended cells from guinea pig epidermis (43), respectively. The effect of retinyl acetate on ionophore induction of envelopes in the rat cell lines of the present study is shown in Table I. At 4 $\mu\text{g/ml}$, this

agent reduced envelope competence in the bladder cells generally about fourfold but was not effective in the pituitary or thyroid cells. This result was not due to a toxic effect on the bladder cells since colony-forming efficiencies were not significantly affected upon subculture. At a concentration of 0.4 $\mu\text{g/ml}$, envelope competence in the bladder cells was halved (26%). In these experiments, hydrocortisone (0.4 $\mu\text{g/ml}$), which increases envelope competence in malignant human epidermal keratinocytes (31), had no effect when added alone and did not antagonize the inhibitory effect of retinyl acetate.

The cells grown from all three epithelia synthesized keratin proteins. As shown by PAGE (Fig. 3a), cellular proteins insoluble in nonionic detergent had mobilities corresponding to a molecular weight range of ~40,000–56,000. The patterns derived from the three epithelial types exhibited prominent bands at 56,000 and 52,000/50,000 mol wt, but reproducible large differences among them in staining intensity of bands with greater mobility were evident in repeated experiments. With continued propagation of each line, the patterns were stable, being virtually identical at least from passages 4–14. The identification of these proteins as keratins was confirmed by their reactivity with rabbit antiserum raised against total keratin of human stratum corneum (Fig. 3b). The relative intensities of staining for protein (amido black) and antigen-

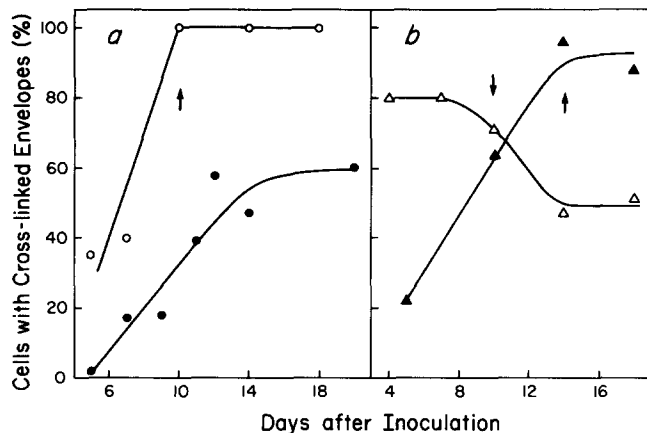


FIGURE 2 Competence in cross-linked envelope formation as a function of days after inoculation. At the times indicated, cultures were trypsinized and treated first with ionophore and then with detergent and reducing agent prior to scoring of cross-linked envelopes. *a* gives the results for pituitary cells in primary culture (●) and after three passages (○). *b* shows thyroid (▲) and bladder cells (△) after three passages. Representative single experiments are given for pituitary, thyroid, and bladder lines, each repeated at least twice, while the points shown for pituitary primary cultures are a compilation of four experiments performed identically. Approximate times (± 1 d) at which the cultures became confluent are indicated by arrows except for primary pituitary, unable to reach confluence due to vigorous fibroblast growth. The decrease in envelope competence of bladder cells after confluence was correlated with considerable desquamation, leaving noticeable gaps in the epithelial pavement that were not filled in.

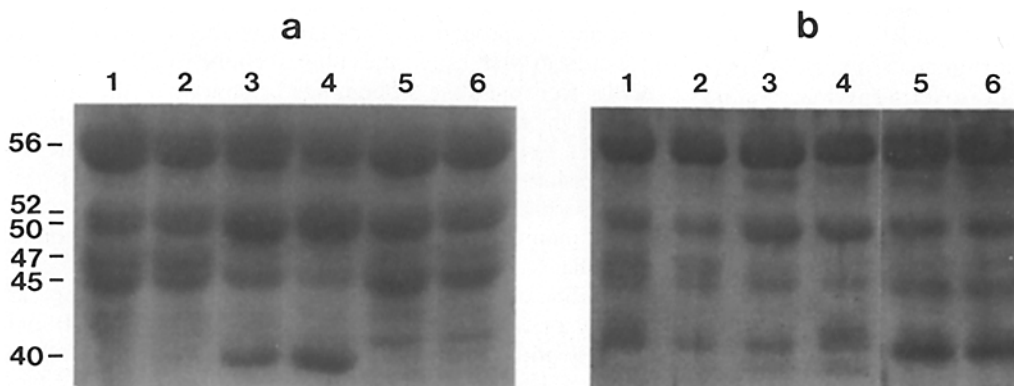


FIGURE 3 Profiles of keratin proteins separated by detergent gel electrophoresis. After transfer to nitrocellulose, the proteins were (a) stained with amido black or (b) treated with antikeratin antiserum and stained by the immunoperoxidase technique. Lanes 1 and 2 (thyroid), 3 and 4 (bladder), and 5 and 6 (pituitary) show extracts from confluent cultures of passage 14 (odd numbers) or 4 (even numbers). At the far left are shown the apparent molecular weights ($\times 10^{-3}$) of the amido black stained proteins, using BSA, rabbit muscle actin, glyceraldehyde phosphate dehydrogenase, and glycerophosphate dehydrogenase as molecular weight standards. Approximately 6 or 3 μg of protein was applied per lane in *a* or *b*, respectively. When considerably lower amounts of protein were applied in *a*, the band at 52,000/50,000 mol wt was resolved into a doublet in the thyroid samples but appeared as a single band of 50,000 mol wt in the bladder and pituitary samples.

icity (immunoperoxidase) differed somewhat, especially for the lowest molecular weight bands. In particular the 40,000-mol-wt protein visible in the bladder cell extract corresponds in both its mobility and weak antigenicity (41) to the 40,000-mol-wt keratin seen in human bladder (42) as well as other epithelial cells (23). A protein of ~41,000 mol wt was only faintly visible when stained with amido black, but stained relatively more strongly with the antiserum, especially in lanes 5 and 6 (pituitary). As shown in Table I, keratin accounted for 20–50% of total cell protein. The content changed little upon vitamin A treatment, although preliminary experiments indicated some effects of added retinyl acetate on the relative intensities of the bands. By contrast, little if any keratin was obtained from fresh rat pituitary tissue.

Growth and Envelope Competence in Cells Derived from Other Nonkeratinized Epithelia

Epithelial outgrowths from explants of minced tracheal, endometrial, vaginal, mammary, and seminal vesicle tissue, passaged with feeder layer support under conditions giving optimal growth of the pituitary cells, all gave colony-forming efficiencies in the range of 0.1–0.5%. It appeared that establishment of continuous lines occurred routinely upon serial passage of each cell type inasmuch as colony-forming effi-

TABLE I
Influence of Retinyl Acetate on Envelope Competence and Keratin Content*

Epithelial origin	Retinyl acetate concentration $\mu\text{g}/\text{ml}$	Keratin content†		Cells with cross-linked envelopes	
		Exp. 1	Exp. 2	Exp. 1	Exp. 2
		%		%	
Bladder	0	26	34	63	52
	4	22	38	3	12
Thyroid	0	33	53	73	65
	4	36	45	68	88
Pituitary	0	44	46	100	79
	4	40	32	100	84

* Values reported are for two independent experiments (Exp. 1, Exp. 2). Retinyl acetate or dimethylsulfoxide alone (0.1% final concentration) was added 4–7 d after the cultures were inoculated and the cells were harvested after an additional 5 d.

† Keratin content is expressed as $100 \times$ the ratio of Triton-Tris buffer-insoluble protein to total cell protein.

TABLE II
Dependence of Envelope Competence on Degree of
Confluence*

Epithelial origin	Cells with cross-linked envelopes	
	Sparse culture	Confluent culture
	%	
Vaginal	90	100
Endometrial	72	73
Tracheal	64	89
Seminal vesicle	17	74
Mammary	<1	86

* Values reported are averages of two independent determinations.

ciency rose to 10% or higher by the fifth passage, although several weeks elapsed between each of the first two endometrial cell passages due to relatively slow growth initially. This finding thus seems to reflect a general property of rat cells not limited to fibroblasts (22) or those originating from stratified squamous epithelia as observed previously (11). Outgrowths of prostate explants also gave tightly packed epithelial colonies in secondary culture but were not passaged further.

A survey was undertaken of cross-linked envelope formation in secondary cultures of each epithelium. Colonies of moderate size were treated with the ionophore X537A directly in surface culture. Upon addition of sodium dodecyl sulfate and dithioerythritol, all five cell types were found to produce abundant envelopes. Those observed in prostate cells are illustrated in Fig. 1*d*. When the experiments were performed quantitatively using disaggregated cells, shown in Table II, a majority of cells in confluent endometrial, vaginal, tracheal, and seminal vesicle cultures were envelope competent, while in sparse culture (colonies averaging 50 cells or less) the seminal vesicle and especially mammary cells were considerably less competent than the other types. This behavior was a stable property of each culture type and did not change at later passages. Fibroblasts derived from several rat tissues were consistently unable to form envelopes in sparse cultures or at confluence upon ionophore treatment.

DISCUSSION

The epithelial cells cultivated in this work exhibited substantial synthesis of cross-linked envelopes, a marker heretofore associated only with keratinocytes (8). Differences among the cells were evident, however, in the regulation of this differentiation property as first noted with epidermal and esophageal keratinocytes (11). Low ability to form envelopes was not associated with lack of stratification and squame formation since small (or large) bladder and endometrial colonies were not visibly stratified, while at least some of those in pituitary and thyroid cultures exhibited obvious squames. Thus, competence in some of the rat epithelial cell types may be regulated as a function of growth, whereas in others the regulation appears to be independent of growth.

Pituitary, thyroid, and bladder epithelial cells, the types studied in most detail, were distinguishable by their vitamin A sensitivity of envelope formation and by their electrophoretic profile of keratin polypeptides. The suppression of envelope competence in bladder cells by added retinyl acetate is consistent with suppression by the vitamin of stratified squamous differentiation in cultures of chick skin (3). Cellular keratin content was similar to that observed in human epi-

dermal keratinocytes (35) and was not altered by vitamin A treatment. Hence, the effects of the vitamin on envelopes are quantitative while those on keratins appear to be qualitative (4).

Except for vaginal epithelium, the tissues used here normally contain few if any keratinocytes, but can undergo squamous metaplasia *in vivo*. Thus, cultivation on plastic in this system provides conditions suitable for the growth of keratinocytes and also appears to select for keratinocyte character in other cell types, a process resembling squamous metaplasia. This appears to be the most likely explanation for the growth of keratinocyte-like cells from human tracheal and bronchial tissue (7, 19) as well as from rat trachea studied here.

Our observations on propagation of rat pituitary cells are consistent with the difficulties in retaining original differentiated function experienced by many investigators (36) and indicate that one reason for loss of secretory function upon passage is that the epithelial cells that grow express keratinocyte properties. Colonies with this behavior were obtained from disaggregated rat pituitary with at least as high efficiency as that from stratified squamous epithelia (epidermis, esophagus, and vagina), and cells in the initial outgrowths at the periphery of pituitary explants could form envelopes. Since we found few if any cells in the intact pituitary (or endometrial or mammary epithelia) to be competent in envelope formation, these observations suggest that a significant fraction is capable of squamous metaplasia. Small regions of squamous cells are sometimes observed in human pituitary, representing metaplasia or embryonic rests (13, 24), but the only analogous regions of rat pituitary are occasional cysts lined by columnar epithelium (25). In contrast, rat thyroid commonly contains epithelial cysts lined by stratified squamous epithelium (38). Regions of squamous cells derived from metaplasia of follicular cells (5, 16) are rare but well documented in human thyroid.

Squamous metaplasia occurs *in vivo* in a variety of epithelia as a result of hormonal, chemical, mechanical, or pathological stimuli, but its histogenesis and the intracellular control mechanisms involved remain matters of considerable uncertainty. In the rat, vitamin A deprivation, for example, produces keratinization of numerous epithelia including bladder, trachea, female genital tract, prostate, and seminal vesicle but not thyroid or pituitary (40). This syndrome is readily reversed upon readdition of vitamin A to the diet, but reversibility in general may depend upon the nature of the metaplastic stimulus and the properties of the target epithelium. Hence, it is not clear to what extent the cultured epithelial cells retain the ability to express the differentiated character of the original epithelium under suitable conditions. Further work will be required to discern whether the effect of retinyl acetate on suppression of envelope competence in the bladder cells represents some such retention and which other factors contribute to maintenance of proper function in the nonsquamous epithelia.

Regions of squamous epithelium have been suggested as sources of craniopharyngioma of the pituitary (13, 24) and primary squamous cell carcinoma of the thyroid (12) in humans. These hypotheses are attractive in view of the established association of squamous cell carcinoma with squamous metaplasia of human lung (21) and cervix (15) which may apply to other epithelia as well. It is unclear, however, whether the neoplastic progression is initiated in the original epithelial cell type in a region subsequently undergoing metaplasia or

in a keratinocyte-like cell during (or even after) the metaplastic process. Reports that rat pituitary (2), mouse prostate (18), and hamster lung (32) undergo squamous metaplasia upon treatment with carcinogenic polycyclic aromatic hydrocarbons (benzo[a]pyrene, 3-methylcholanthrene) emphasize the importance of studying both the keratinocyte character in cells derived from these sites and the mechanism generating its expression.

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