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Effect of growth environment on spatial expression of involucrin by human epidermal keratinocytes

F. M. Watt[1], P. Boukamp[2], J. Hornung[2], and N. E. Fusenig[2]

[1] Molecular Cell Biology Laboratory, Kennedy Institute of Rheumatology, 6 Bute Gardens, London, W6 7DW, England
[2] Division of Differentiation and Carcinogenesis in vitro, Institute of Biochemistry, Deutsches Krebsforschungszentrum (DKFZ), D-6900 Heidelberg, FRG

Summary. Involucrin, the major protein precursor of the cornified envelope, is expressed during terminal differentiation of human keratinocytes, both in vivo and in vitro. In epidermis, the onset of synthesis is several layers above the basal layer, but in stratified cultures of keratinocytes on tissue culture plastic involucrin synthesis begins in the first suprabasal layer. To investigate the reason for this premature expression, the distribution of involucrin was studied in epidermis from different body sites, in organotypic cultures and in transplants of keratinocytes onto nude mice. We found that premature expression was not associated with poor morphological differentiation, because involucrin synthesis began immediately above the basal layer even when distinct basal, spinous, granular and cornified layers were formed in organotypic cultures recombined with dermis. The site of involucrin expression in culture did not depend on the number of cornified layers present. The only conditions which resulted in an upward shift in the site of synthesis were in 3-week old transplants on nude mice. We conclude that the site of onset of involucrin synthesis is not determined by the degree of morphological differentiation of the tissue, and discuss other factors which may be involved.

Key words: Keratinocytes – Involucrin – Organotypic cultures – Transplants

In normal human epidermis proliferation is largely restricted to the basal layer and keratinocytes undergo terminal differentiation as they migrate through the upper cell layers. Maintenance of normal tissue architecture depends on the existence of a steady state between the rate of production of new cells in the basal layer and the rate of shedding of cells at the final stage of terminal differentiation from the outermost layers [20].

In vivo studies of how terminal differentiation is controlled are necessarily limited, and so a number of in vitro models have been developed. Keratinocytes have been grown on different substrata; submerged or exposed to air; with or without 3T3 feeder cells or pieces of dermis; in the presence or absence of serum; and with a range of medium supplements [reviewed in 9, 13, 17, 21]. Most cultures retain the essential properties of intact epidermis, in that cell proliferation takes place in the basal layer in contact with the substratum, cells undergo some features of terminal differentiation as they move through the suprabasal layers and they are eventually shed from the culture surface. However, the histological appearance of the cultures varies, and distinct basal, spinous, granular and cornified layers are only formed when the growth conditions approximate a normal epidermal environment [9, 15, 16].

Late in terminal differentiation an insoluble protein envelope, the cornified envelope, is laid down underneath the plasma membrane. Keratinocytes with cornified envelopes are known as squames. In normal epidermis there are several layers of squames, but in culture on plastic cells containing envelopes tend to slough off into the medium, and cornified layers do not accumulate [12, 24]. In epidermis the squames have usually lost their nuclei and the cornified layers are termed orthokeratinized. However, in cultures on plastic [12] and in epidermis of some body sites, envelopes may form before the nuclei are lost and layers of nucleated squames are said to be parakeratotic.

The cornified envelope is assembled by transglutaminase-catalyzed cross-linking of several pre-

Offprint requests to: Dr. F. M Watt, Imperial Cancer Research Fund, P.O.Box 123, Lincoln's Inn Fields, London WC2A 3PX, England

cursor proteins, the most abundant of which is a cytoplasmic protein called involucrin [22, 23]. Keratinocytes synthesize involucrin some time before the cornified envelope is formed. In intact epidermis, involucrin synthesis begins when keratinocytes have migrated about one- to two-thirds of the distance from the basal layer to the tissue surface [22]. However, in culture on plastic or on submerged collagen gels the onset of synthesis is almost always immediately above the basal layer [3, 25]. This does not reflect an intrinsic defect in cultured keratinocytes, but appears to be a response to the culture environment, because when cultures are grafted onto nude mice the site of involucrin synthesis shifts upwards to its normal in vivo position [2].

The aim of the experiments described in this report was to see whether the site of involucrin expression in natural and cultured epidermis depends on tissue architecture and accumulation of cornified layers. We have compared the distribution of involucrin in the epidermis of skin samples from different body sites and in keratinocytes grown under conditions that improve morphological differentiation, including the formation of well-developed granular and cornified layers.

Materials and methods

Keratinocyte isolation and culture medium

Keratinocytes were isolated from full thickness adult human skin (usually from arm or thigh), obtained after surgical excision. Skin samples were freed of subcutaneous tissue and 1 cm^2 pieces were incubated in 0.2% trypsin in calcium- and magnesium-free phosphate-buffered saline (PBS) at 4° C for 1–2 days. The epidermis was then peeled from the dermis and cells of the lower layers detached by vigorous pipetting. The dermis was scraped to remove adherent basal keratinocytes and these were combined with the other epidermal cells. Keratinocytes were plated at high density ($1.5-4\times10^6$ cells per 60 mm dish; Falcon) without a 3T3 cell feeder layer and grown at 37° C in a humid atmosphere of 5% CO_2 in air. First or second passage keratinocytes were used in the organotypic cultures and transplantation experiments.

The culture medium consisted of 1 part Ham's F12 medium plus 3 parts Dulbecco's modified Eagle's medium, supplemented with 1.8×10^{-4} *M* adenine, 5% foetal calf serum, 5 μg/ml insulin, 0.5 μg/ml hydrocortisone, 10^{-10} *M* cholera toxin and 10 ng/ml epidermal growth factor [32].

Organotypic cultures

Keratinocytes were seeded onto glutaraldehyde-fixed (4% for 1 h) collagen gels cast in silicone chambers (Renner, Dannstadt, FRG), as described in detail elsewhere [6, 11]. The chambers were placed in "Stanzen" petri dishes (Greiner, Nürtingen, FRG) so that the cultures were raised to the air-medium interface and fed from below. In some experiments, keratinocytes were recombined with dermis: 1 cm^2 of de-epithelialised adult human dermis was placed underneath each collagen gel as described previously [9, 18]. Cultures were harvested 7 to 21 days after plating for histology or cryostat sectioning.

Transplantation of keratinocytes onto nude mice

Organotypic cultures (i.e., keratinocytes grown on collagen gels in silicone chambers) were transplanted onto the back-muscle fascia of 5- to 7-week-old nude mice (Balb/c nu/nu), exposed by interscapular incision as described [6, 31]. The transplantation chambers were covered with a second, closed, silicone chamber (Renner, Dannstadt, FRG) to prevent desiccation, infection and growth of host epidermis into the transplant. The whole transplantation unit was held in place by closing the surrounding skin with wound clips.

Immunofluorescence

Skin for immunofluorescence staining was taken from adult forearm or leg or from foreskin of children (6–14 years old). Keratinocyte cultures, transplants and pieces of skin were embedded in Tissue Tek O.C.T. (Lab Tek Products, Naperville, Tenn., USA) and snap-frozen in liquid nitrogen. Cryostat sections (5–7 μm) of the specimens were air-dried and incubated with rabbit antiserum to involucrin [29], followed by affinity-purified fluoresceinated goat anti-rabbit IgG (Miles-Yeda, Rehovot, Israel). Incubations were carried out at room temperature for 30 min and sections were washed thoroughly in PBS between incubations. After a final rinse in 20 m*M* Tris-HCl (pH 8.5) sections were mounted in Aqua-Mount (Lerner Laboratories, New Haven, Conn., USA) and examined with a Zeiss inverted microscope equipped with epifluorescence optics. Control sections incubated with preimmune serum and second antibody showed low levels of non-specific staining.

Paraffin wax sections of parallel specimens were incubated with anti-involucrin for immunofluorescence or stained with haematoxylin and eosin (H and E) for histological observation. The distribution of involucrin-positive cells was the same in frozen and wax sections, although, as noted previously [25], involucrin was concentrated at cell boundaries in frozen sections and was uniformly distributed throughout the cytoplasm in wax sections.

Results

Previous studies have shown that the onset of involucrin synthesis in normal epidermis is a variable distance through the spinous layers [22]. We therefore investigated the site of involucrin expression in epidermis from body sites chosen most frequently as a source of cultured keratinocytes.

In normal orthokeratinized body skin, involucrin expression began in the upper spinous layers, coincident with cell flattening (Fig. 1a, b). However, the onset was earlier in foreskin. In the orthokeratinized epidermis of the outer foreskin, the zone of cells that stained positive for involucrin was broader than in body skin, starting 2–3 cell layers above the basal layer, in a region where the cells were still cuboidal (Fig. 1c, d). In the parakeratotic epidermis of the inner surface (Fig. 1e) involucrin expression began even earlier: only the basal layer and one or two suprabasal layers were unstained (Fig. 1f).

In contrast to these results, the onset of involucrin synthesis in stratified cultures of keratinocytes grown on tissue culture plastic or submerged collagen gels is

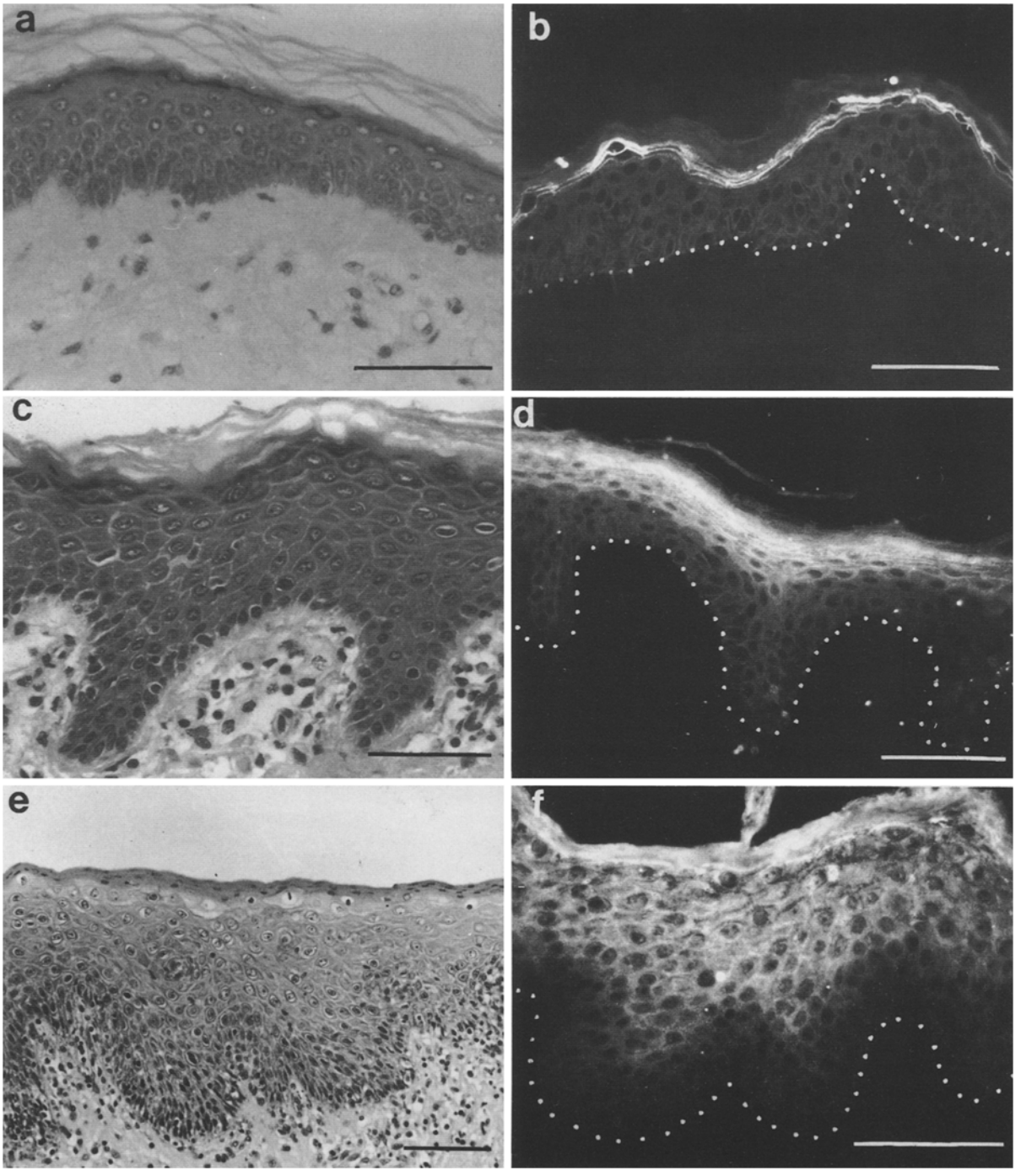

Fig. 1 a–f. Sections of epidermis from different body sites. **a**, **c**, **e** HE staining of wax-embedded material. **b**, **d**, **f** Immunofluorescence staining of unfixed frozen sections with antiserum to involucrin. *Dotted lines* indicate position of basement membrane. **a**, **b** Adult forearm; **c**, **d**, outer side of infant foreskin; **e**, **f**, inner side of same foreskin as in **c**, **d**. *Scale bars*, 100 μm

the same, regardless of body site or age of donor ([3, 25]; P. Boukamp and F. M. Watt, unpublished observations). Only cells in the basal layer, and occasional cells in the layer above, lack involucrin. In such cultures, morphological differentiation is poor: cells in all layers are flattened and there are no distinct granular or cornified layers. We therefore grew keratinocytes from adult body skin under conditions in which the histological appearance of the cultures was improved, to see whether this would lead to an upward shift in the site of onset of involucrin synthesis.

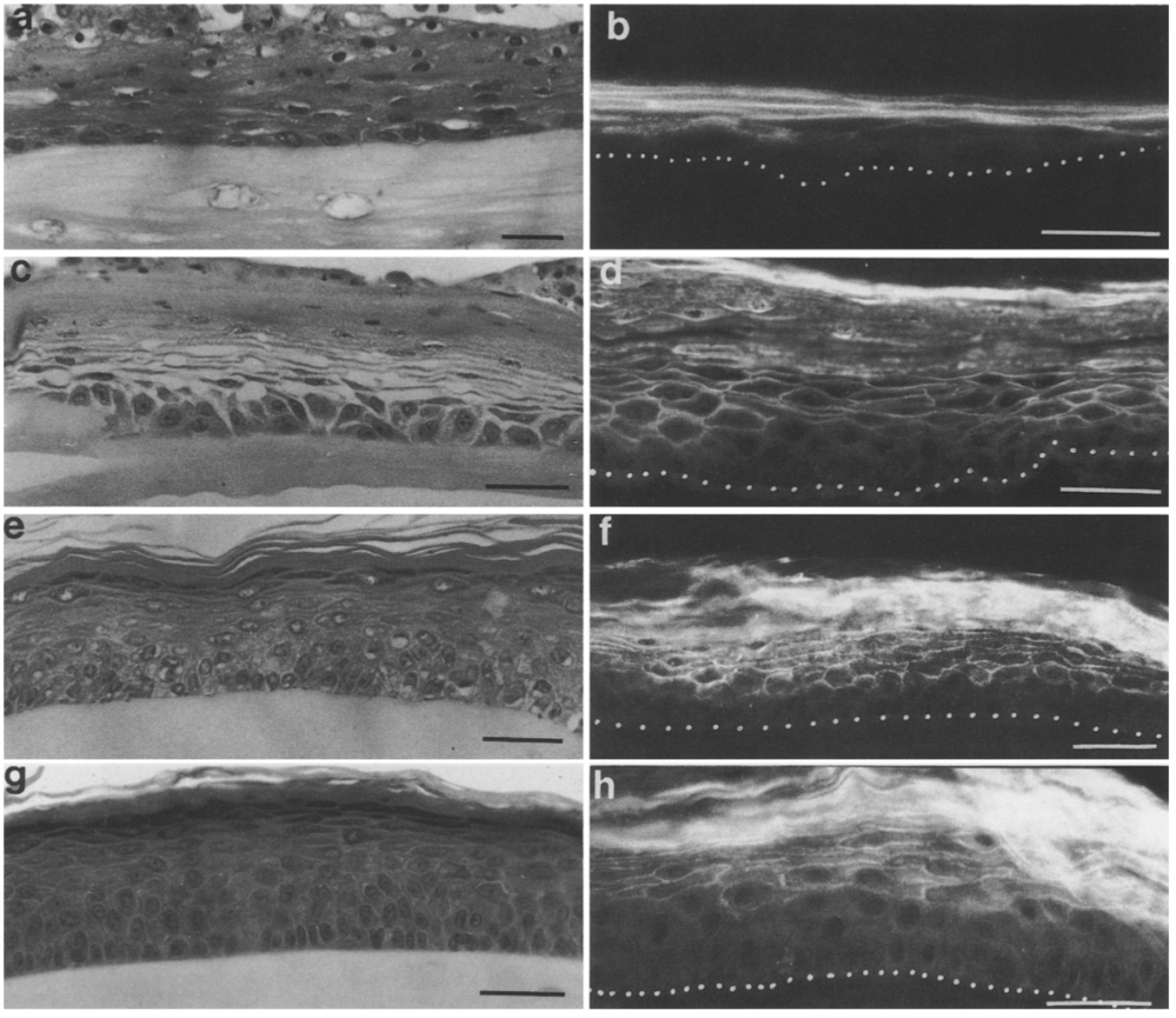

Fig. 2a–h. Sections of cultures and transplants of human keratinocytes isolated from adult body skin. **a**, **c**, **e**, **g** HE staining of wax-embedded material. **b**, **d**, **f**, **h** Immunofluorescence staining of unfixed frozen sections with antiserum to involucrin. *Dotted lines* indicate boundary between epidermis and collagen. **a**, **b** Organotypic cultures 2 weeks after plating; **c**, **d**, organotypic cultures 10 days after plating and 8 days after recombination with dermis from adult body skin; **e**, **f**, cultures on collagen gels 7 days after transplantation onto nude mouse; **g**, **h**, cultures on collagen gels 3 weeks after transplantation. *Scale bars*, 50 μm

Cultures of keratinocytes on raised collagen gels exposed at the air-liquid interface (organotypic cultures) had more layers and more cells with cornified envelopes than cultures on plastic or submerged collagen gels (Fig. 2a), although they still lacked a granular layer and most of the squames were nucleated. In these, as in submerged cultures, only the basal layer, and occasional cells in the layer above, lacked involucrin (Fig. 2b).

When organotypic cultures were recombined with homologous dermal mesenchyme, there was a further improvement in their histological appearance. The basal cells were cuboidal instead of flattened; there was increased stratification with more layers of nucleated squames (Fig. 2c). However, in these cultures there were again only one or, occasionally, two involucrin-negative layers and involucrin was expressed by cells before they had flattened completely (Fig. 2d).

When keratinocytes on collagen gels were transplanted onto nude mice their morphology resembled normal epidermis more closely [5]. After 7 days a thin

layer of granular cells was clearly visible, as were several layers of anucleate squames (Fig. 2e). Even so, only the basal layer and some cells in the layer above lacked involucrin (Fig. 2f). Three weeks after transplantation the arrangement of the living cell layers had become more organized, and the only clear morphological difference between the grafts and intact epidermis was the straight basal border line along the collagen gel (Fig. 2g). Involucrin expression at this time had shifted upwards: at least three cell layers were involucrin-negative; and the onset of synthesis was correlated with cell flattening, as in orthokeratinized epidermis of adult body skin (Fig. 2h).

Discussion

Synthesis of involucrin marks a stage in terminal differentiation after keratinocytes have stopped dividing, but before formation of the cornified envelope. Synthesis begins a variable distance through the spinous layers of epidermis [22], but in cultures of epidermal cells on plastic the onset of involucrin synthesis is immediately above the basal layer [3]. Using involucrin as a marker of suprabasal, terminally differentiating, keratinocytes it has been shown that movement upwards from the basal layer is not required for terminal differentiation, but that the two processes are normally linked through changes in cell adhesiveness during terminal differentiation [26, 28].

In this report we have investigated possible reasons for the premature expression of involucrin in culture. We have shown that an overall improvement in culture morphology, resulting in distinct spinous, granular and cornified layers, does not lead to an upward shift in the onset of synthesis. In organotypic cultures recombined with mesenchyme and in transplants, but not in cultures on plastic, filaggrin and the high-molecular weight keratins characteristic of epidermal differentiation are synthesized ([7, 9, 10]; P. Boukamp et al., in preparation). Thus, expression of involucrin is also regulated independently of other biochemical markers of keratinocyte terminal differentiation.

In foreskin epidermis, involucrin expression was earlier in parakeratotic than in orthokeratinized regions. However, the site of involucrin synthesis in cultures did not depend on whether the squames were anucleate, or on the number of cornified layers present. Thus, the distribution of involucrin is not tightly linked to ortho- or parakeratinization or to envelope formation. This agrees with previous findings: stratified squamous epithelia that lack cornified envelopes still synthesize involucrin several layers above the basal layer [3]; and vitamin A can suppress envelope formation without affecting the involucrin content of the cells [14].

Involucrin expression is correlated with an increase in cell size, both in vivo [3] and in vitro [27]. It is often associated with a change in cell shape from cuboidal to flattened (for example, Figs. 1b, 2h); however, this relationship is not absolute (see Figs. 1f, 2f). At present it seems unlikely that changes in cell size and shape are the sole factors determining the onset of involucrin synthesis; but they may play a secondary role.

Another possibility is that premature involucrin expression is linked to altered tissue homeostasis, as observed in hyperproliferative and diseased epidermis. This is suggested by the observations that involucrin is synthesized earlier in psoriatic epidermis than in normal adult body skin [4, 19] and that keratinocytes in culture are hyperproliferative by several different criteria [1, 7, 8, 30]. Three-week-old keratinocyte transplants, in which involucrin expression is shifted upwards, may represent a situation that is intermediate between cultured and normal epidermis, in terms of growth fraction and proliferation rate.

In conclusion, the site of involucrin expression is not closely correlated with envelope formation, cell flattening or orthokeratinization in general. There is circumstantial evidence that premature localization may reflect a hyperproliferative state, and if this is the case, localization of involucrin may be a sensitive marker for epidermal homeostasis. Current investigations into the time interval between cessation of proliferation and initiation of involucrin synthesis should shed more light on this question (R. Dover and F. M. Watt, in preparation).

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