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Segregation of keratinocyte colony-forming cells in the bulge of the rat vibrissa

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ABSTRACT **The epidermis and its related appendages such as the hair follicle constitute the epithelial compartment of the skin. The exact location and distribution of the keratinocyte colony-forming cells within the epidermis or its appendages are unknown. We report that in the rat vibrissa, keratinocyte colony-forming cells are highly clustered in the bulge-containing region. Approximately 95% of the total colonies formed in culture from fractionated vibrissae were in this location and fewer than 4% were located in the matrix area of the follicle. Finer dissection of the bulge-containing region located the colony-forming cells in the small part containing the bulge itself. The segregation of keratinocyte colony-forming cells in the bulge confirms the hypothesis that the bulge is the reservoir of the stem cells responsible for the long-term growth of the hair follicle and perhaps of the epidermis as well.**

Hairs are specialized epidermal appendages which are characteristic of mammals. The hairy coat plays a significant role in temperature regulation, and also functions as a camouflage. Some hairs, such as the whiskers, have evolved as tactile organs and are important for animal behavior. In the human, the hairs are not as crucial and are generally viewed more as a cosmetic advantage. Nevertheless, the hair follicle provides a reservoir of keratinocytes which can be recruited to reepithelialize a skin defect (1). Hairs develop from the primitive epidermis during fetal life (2, 3). The inductive signals are believed to be released by the mesenchymal cells that constitute the dermal papilla (4–6). The exact nature of these signals is still unknown, although epimorphin, a membrane protein reported as essential for epithelial morphogenesis, may play a role (7). Follicular keratinocytes acquire a specific program of proliferation and differentiation as illustrated by the expression of the hair keratin genes (8–10). One of the most striking features of the hair is its carefully regulated growth cycle. Each cycle is a succession of three phases. The active growth phase (anagen) is followed by a regression phase (catagen) and then by a resting phase (telogen) after which growth resumes. The factors regulating these different phases are not understood. Cells with enough proliferative capability must exist in the hair follicle to provide differentiated progeny for the life time of the animal (11). The exact location of these cells within the hair follicle is not known with certainty. The follicular stem cells have long been thought to reside in the matrix where an intense proliferative activity takes place during the anagen phase (12–14). Particularly insightful work on this problem by Cotsarelis *et al.* (13) led to the discovery that follicular cells retaining [^{3}H]thymidine were located in the bulge region close to the insertion of the arrector pili muscle. These data, along with previous histological findings (2, 15–18), make the bulge a tempting candidate for the site of the follicular stem cells. Cultivation of follicular keratinocytes from the rat vibrissa has been reported (5), but the colony-forming ability of the keratinocytes from the different regions of the vibrissa has not been examined in detail.

MATERIALS AND METHODS

Rats. Fischer 344 (inbred) rats were obtained from the Curie Institute animal facility. Long Evans (inbred) rats were from the Centre National de la Recherche Scientifique animal service (Orléans, France). In all cases 3- to 5-week-old females were used.

Preparation of Vibrissae. Rats were killed by carbon dioxide asphyxiation. The upper lip containing the two vibrissa pads was cut out and its inner surface was exposed. Each selected vibrissa was then microdissected under a binocular microscope after its position was recorded according to Oliver's whisker map (15). The subcutaneous fat and the connective tissue surrounding its capsule were carefully removed. The vibrissa was then pulled away from the pad by holding its neck with a fine forceps. It was then kept in sterile culture medium. A transverse incision was made on the collagen capsule surrounding the cavernous sinus just above the hair bulb. The lower part of the capsule was then carefully pulled away from the bulb; special care was taken at this stage to keep the dermal papilla in place. A longitudinal incision was then made on the remaining upper part of the capsule. The epithelial core with the thin dermal sheath surrounding it was then carefully detached from the capsule. The follicle was photographed and was then cut into four fragments. The first transversal cut was made just above the hair bulb, and the second was made at the site of insertion of the nerve fibers, which is proximal to the site of inferior enlargement of the outer sheath (19). The third cut was made just below the sebaceous glands.

The fragments were arranged in the original order of the follicle and photographed. Each fragment was then individually transferred into a 35-mm dish containing 1 ml of collagenase/dispase (1 mg/ml; Boehringer Mannheim) and incubated for 30 min at 37°C. The epithelial cells were then teased away from the thin dermal sheath with fine needles. At that stage the dermal papilla was detached from the matrix but was left to incubate in the dish. A solution of trypsin (0.05%) was then added and the fragments were further incubated till the dissociation was completed, usually by 1 hr.

Histology. Vibrissae were fixed with 3.7% formaldehyde in phosphate-buffered saline or with methanol/acetone (1:1, vol/vol). They were then embedded in paraffin and 7-μm sections were stained with a combination of hematoxylin, Ponceau S, and alcian blue (20). Vibrissae or fragments of vibrissae were snap-frozen in liquid nitrogen and stored at −80°C. Frozen sections (8 μm) were obtained with a Cryocut 1800 (Reichert) whose cutting-chamber temperature was set at −30°C.

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Immunoperoxidase Staining. Cultures were stained by the peroxidase–antiperoxidase technique according to standard procedures and manufacturers' guidelines. Antibodies were from DAKO and from Boehringer Mannheim (anti-cytokeratin pan). Endogenous peroxidase activity was quenched by treating samples with 1% hydrogen peroxide.

Cell Count. Cells were isolated from the different parts of nine vibrissae in anagen phase as described above. Dermal papilla was removed from the P1 part. In most cases, the thin dermal sheath was not removed from the P3 fragment. Aliquots of cell suspension were centrifuged onto glass slides for 5 min at 800 rpm (Cytospin 3; Shandon, Pittsburgh). Cells were counted on a Zeiss microscope equipped with phase-contrast optics. An average of four aliquots per fragment were used for cell counts. The total number of cells per fragment was obtained by averaging the number of cells in the aliquots and then correcting it to the original volume of the cell suspension.

Cell Culture. Keratinocytes were cultivated on a lethally irradiated feeder layer of 3T3-J2 cells according to Rheinwald and Green (21). The culture medium, a 3:1 mixture of the Dulbecco–Vogt modification of Eagle's medium and Ham's F12 medium, was supplemented with 10% fetal bovine serum (22, 23). Human recombinant epidermal growth factor, kindly provided by Pablo Valenzuela (Chiron) was added at 10 ng/ml beginning at the first feeding. The cells were usually fed every 4 days. All experiments were carried out with a single batch of fetal bovine serum (HyClone).

RESULTS

Segregation of Colony-Forming Cells in the Vibrissa. To locate the keratinocyte colony-forming cells, individual vibrissae were dissected from 21- to 28-day-old female rats of either the Fischer 344 or the Long Evans strain. At that age, the vibrissae are in the anagen phase of the hair cycle (20, 24). The capsule was microdissected away from the epithelial structure, which was then photographed (Fig. 1 *Left*). Each vibrissa was then carefully cut into four fragments corresponding to anatomically distinct parts of the follicle (Fig. 1 *Right*): the part containing the matrix was designated as P1, the bulge-containing part was designated P3, the intermediate part P2, and the last part, containing the sebaceous glands, P4. Special care was taken not to remove the dermal papilla when cutting out the matrix area, in order not to damage the matrix cells. Each part was photographed and then incubated briefly in collagenase/dispase. After the dermal sheath was teased apart from the epithelial part with fine needles, trypsin was added in order to obtain a single-cell suspension. For assay of growth capability, the cells were then plated into individual culture dishes containing a feeder layer of lethally irradiated 3T3 cells.

In all vibrissae (n = 38) isolated in the mid- to late-anagen phase from 19 rats, many keratinocyte colonies formed from cells of the bulge-containing region (P3) (Fig. 2). They represented >95% of the total colonies isolated from the vibrissae (Table 1); the remainder of the colony-forming cells were mainly present in P1. Very few colony-forming cells were located in P2 and P4. When a few colonies did form from P4, it was confirmed by examination of the photographs that the cut had failed to separate part of the bulge from P4. The difference in number of colony-forming cells between P1 and P3 cannot be explained by differences in the number of cells present in those locations: counts of total cells liberated from P1 and P3 by trypsinization showed that P1 contained an average of 7000 cells, whereas P3 contained an average of 5400 cells. P2 and P4 fragments each contained an average of 500 cells.

Segregation of the Keratinocyte Colony-Forming Cells in the Bulge. When the individual fragments were located on a histological section of a vibrissa, it was found that the P3 fragment contained the bulge (Fig. 3). In order to locate more precisely the colony-forming cells in P3, this region was further subdivided into three distinct subregions designated P3-1, P3-2, and P3-3 (Fig. 4). Cells were isolated from each subregion and cultivated. Most of the colonies formed from cells isolated from the P3-2 subregion (Fig. 5). This fragment contained the bulge as demonstrated first in the dissecting microscope by the presence of an obvious bulging of the epithelial part of the fragment after its dermal sheath component was removed by the collagenase-dispase treatment and second by histological identification of the bulge on frozen sections made from P3-2 fragments (Fig. 6). Counts of total cells liberated from P3-2 fragments by trypsinization showed that P3-2 contained an average of 2100 cells. In two separate experiments, the colony-forming efficiency of these cells was determined to be 10% and 19%, respectively.

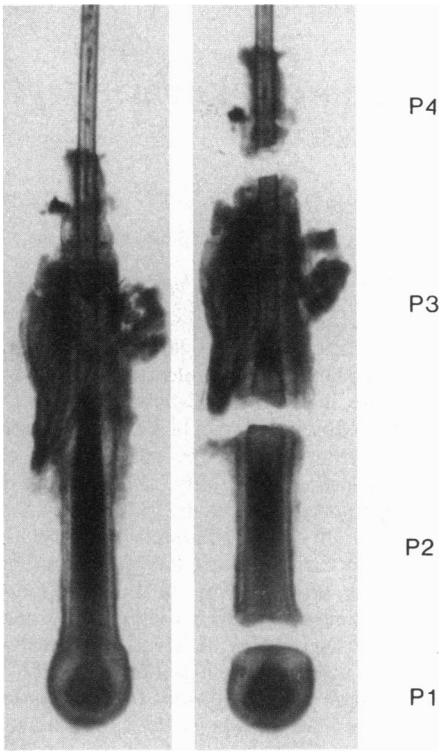

FIG. 1. Microdissection of the bulge-containing region. (*Left*) Aspect of a dissected rat vibrissa (KR4) in anagen phase. It was isolated from row *a*, according to Oliver's map (15), of the right whisker pad of a 21-day-old female Fischer rat. The epithelial core with the thin surrounding dermal sheath was carefully dissected away from the vibrissa capsule. (*Right*) The same vibrissa after it was cut into four fragments. The first transverse cut was made just above the hair matrix and the second was made at the site of insertion of the nerve fibers, proximal to the site of inferior enlargement of the outer root sheath (19). The third cut was made just below the sebaceous glands. Total length of vibrissa follicle is 2.7 mm. P1, matrix-containing part; P2, intermediate part; P3, bulge-containing part; P4, sebaceous gland-containing part.

Morphology of the Epithelial Colonies. The epithelial nature of the cells that formed the colonies was confirmed by immunoperoxidase staining with a monoclonal antibody that recognized an epitope common to all keratins (anti-cytokeratin pan; Boehringer Mannheim). On microscopic observation, keratinocyte colonies isolated from P1 appeared to have a more differentiated appearance than colonies derived from P3, as it was more common to observe large squame-like cells within the colonies formed from P1. Nevertheless, P1 cells could give rise to a few progressively growing colonies, which could be passaged several times.

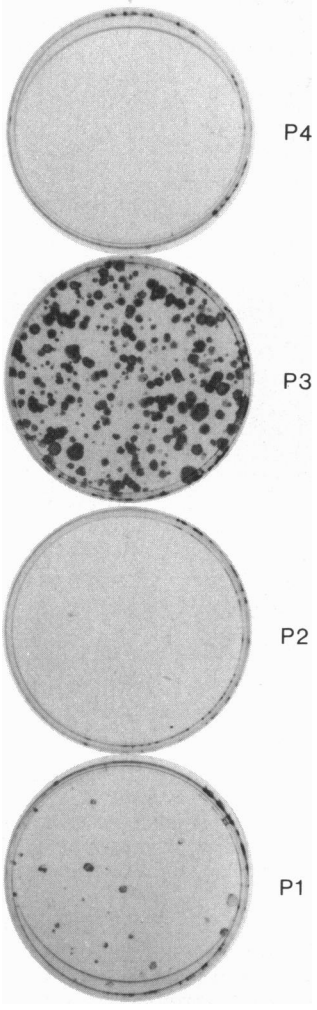

FIG. 2. Segregation of colony-forming cells in the vibrissa. Keratinocytes were isolated from fragments P1–P4 of the vibrissa shown in Fig. 1 and then cultivated for 9 days before they were fixed and stained with 1% rhodamine B. Virtually all colony-forming cells were located in P3.

Large rapidly growing colonies were the rule for cells of P3. These colonies contained mainly small cells, which were highly proliferative, with an average doubling time of 13 hr, an extraordinarily short cycle time in comparison with most epidermal cultures. The range of doubling time was 9–18 hr.

Table 1. Numbers of keratinocyte colony-forming cells isolated from rat vibrissae

	No. of colony-forming cells				
Vibrissa	P1	P2	P3	P4	% in P3/P4
KR4	50	5	366	0	87
KR5	32	28	740	240	94
KR6	52	6	644	66	92
KR7	24	5	1200	0	98
KR8	8	8	600	0	97
KR15a	22	4	1070	20	98
KR15b	18	6	560	0	96
Mean	29.4	8.9	740	46.6	95

Cells were isolated from individual vibrissa in mid- to late-anagen phase and cultivated. Keratinocyte colonies were stained with rhodamine B and counted under a dissecting microscope. P1, matrix-containing part; P2, intermediate part; P3, bulge-containing part; P4, sebaceous gland-containing part. Results of seven experiments are given.

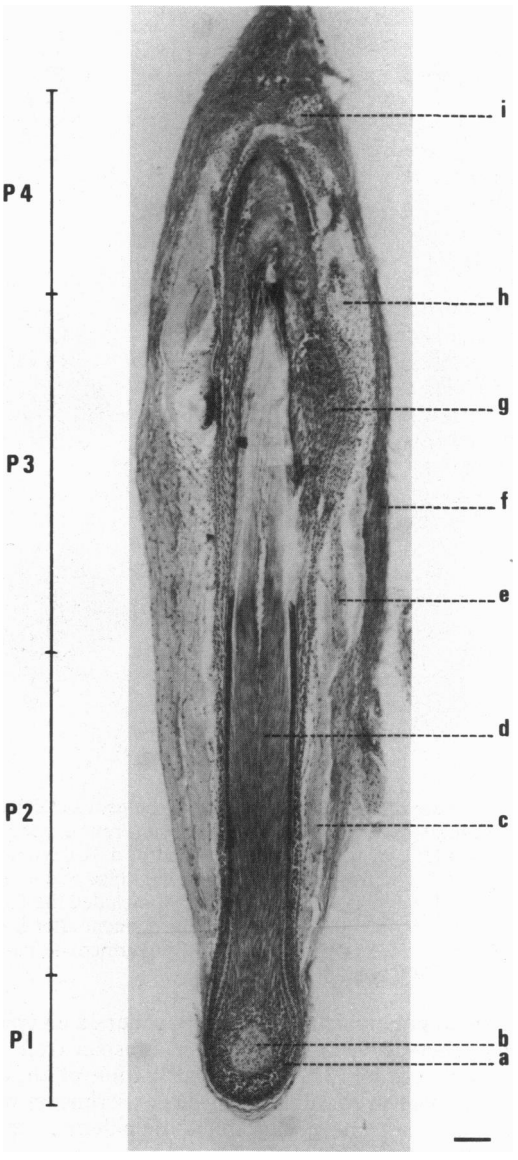

FIG. 3. Histology of a rat vibrissa. A vibrissa was isolated from row *a* of a whisker pad of a 21-day-old female Fischer rat (KR7). Longitudinal section through the bulge-containing area is shown. a, Matrix cells; b, dermal papilla; c, inferior blood sinus; d, hair shaft; e, nerve fibers; f, capsula; g, bulge; h, superior blood sinus; i, sebaceous gland. (Bar = 0.1 mm.)

Differences in doubling time might correspond to individual subpopulations of colony-forming cells. The cells were usually densely packed when the colony was small, but they had a strong tendency to scatter as the colony grew larger. These cells could be passaged several times with no apparent sign of senescence. Nevertheless, an exhaustive study of their growth potential will require their serial cultivation for many more weeks, and as these are rodent cells, they are likely to undergo spontaneous immortalization. Such studies should be performed with human cells, which have a low probability of immortalization. Colonies found in P4 were always progressively growing, like those of P3.

DISCUSSION

The epidermis and its related appendages contain keratinocytes which can form colonies in culture (21), but only a small proportion of them have this ability (25), and the colony-forming cells are heterogeneous in that some have a

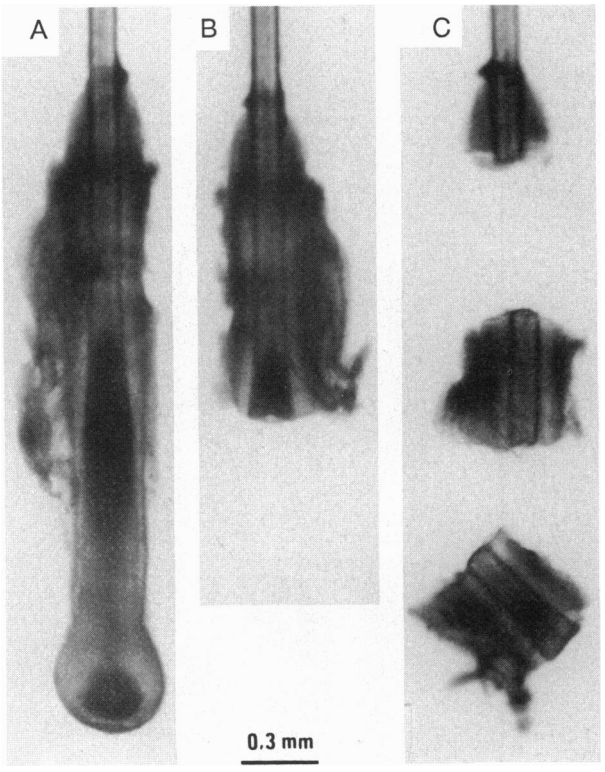

FIG. 4. More precise localization of the bulge. (*A*) Dissected rat vibrissa (YR21) in anagen phase isolated from row *a*, according to Oliver's map (15), of the right whisker pad of a 31-day-old female Fischer rat. (*B*) The fragment containing the bulge region after the inferior part of the vibrissa was removed; it included the P3 and P4 fragments as described in Fig. 1. (*C*) The fragment after it was cut into three parts. P3-1, inferior part; P3-2, intermediate part; P3-3, superior part. P3-2 contained the bulge.

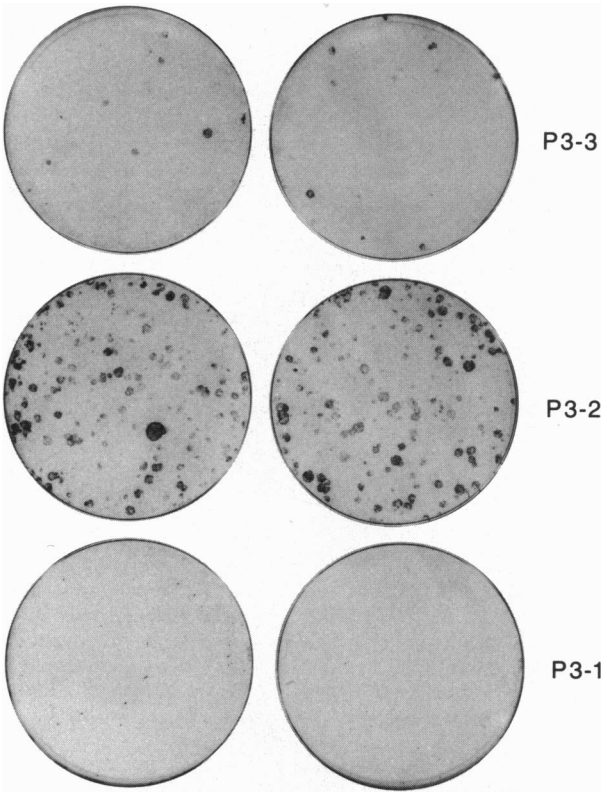

FIG. 5. Segregation of the keratinocyte colony-forming cells in the bulge region. Cells were cultivated from the various fragments shown in Fig. 4. Most colony-forming cells were located in P3-2.

lot of growth potential and are able to generate an epidermis whereas others have a more limited lifespan (ref. 26 and unpublished results). The exact distribution of these cells within the epidermis is unknown, but experiments by Costarelis *et al.* (13) strongly suggested that epidermal stem cells reside in the hair bulge.

The experiments on vibrissa follicles described here revealed a segregation of keratinocyte colony-forming cells in the bulge region of the pilosebaceous unit. The bulge region contained an average of 740 colony-forming cells, or 95% of the total released from follicles: the remaining 5% of the colony-forming cells were mostly located in the matrix. The intermediate region separating the bulge from the matrix area did not contain keratinocyte colony-forming cells when the vibrissa was in the mid-anagen phase of the hair cycle.

The cells that constituted the colonies expressed keratins, confirming their epithelial nature. Although we have not examined in detail the profile of expression of keratins by the differentiated progeny of these colony-forming cells, immunostaining of the colonies with a monoclonal antibody (AE13) that recognizes the human acidic hair keratins in matrix and differentiated follicular cells of frozen sections of rat vibrissa was negative. That result can be explained if the growth ability of the keratinocyte colony-forming cell was favored over its differentiation ability in our experiments.

As the matrix degenerates during the catagen phase of the hair cycle (20, 27), it is likely that the few colony-forming cells that it contained previously are lost. Therefore, to reinitiate a growth phase, a new population of cells must be recruited from the bulge and rejoin the root of the follicle (13). In additional experiments, we found that keratinocyte colony-forming cells could be identified in the intermediate part (P2) only when the vibrissa was in the late catagen phase of its cycle. We interpret this phenomenon as the result of mobilization and migration of some keratinocyte colony-forming cells from the bulge to provide matrix cells for the next anagen phase. This cellular distribution can be viewed as a way to protect and preserve the supply of colony-forming cells, as only few of them will be used for each cycle. In the case of pelage hairs, which have a long resting phase, the recruitment of these cells should occur during the telogen phase of the cycle, but for the vibrissae, which have an extremely short telogen phase (20), the keratinocyte colony-forming cells probably need to be recruited earlier, while the follicle is still in its catagen phase.

Keratinocyte clones with the properties described above could be grown from single cells directly isolated from either the P1 or the P3 region of the vibrissa. This means that the culture conditions using supporting 3T3 cells were adequate to sustain the growth of a single follicular colony-forming cell even after it had been completely removed from the influence of its dermal environment. The growth properties of the colony-forming cells were not improved when dermal papilla cells were used as a feeder layer instead of 3T3 cells. Therefore, the growth potential of the keratinocytes is not uniquely dependent on the fibroblasts of the dermal papilla. However, these fibroblasts may provide the specific recruitment and differentiation signals which are indispensable steps for hair growth *in vivo* (6).

The matrix is the site of an active proliferative activity during the anagen phase (12–14). The small number of matrix keratinocytes able to initiate colony formation seems unlikely to be able to account for all this mitotic activity. We have not observed in P1 the large number of aborted colonies that one should expect to be initiated from a transient amplifying population such as the paraclone (26). Therefore, the matrix must contain transient amplifying cells that cannot be re-

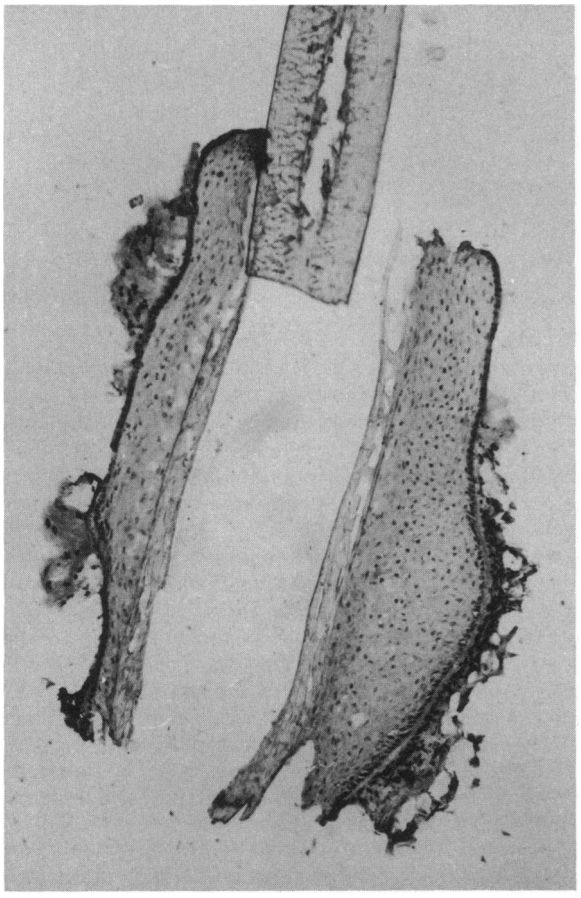

FIG. 6. The P3-2 fragment contains the bulge. Frozen sections (8 μm) were made from a P3-2 fragment which was isolated from the bulge-containing region of a vibrissa of a Fischer rat. The enlargement, as seen in the right part of the section, is typical of the bulge. The central part which slid off during sectioning is the hair shaft. Total length of the P3-2 fragment is 0.65 mm.

vealed by the current cultivation techniques because they have already exhausted most of their growth potential and differentiate before giving rise to a visible colony. There should be many such cells in the matrix if they are to sustain the growing phase of the hair cycle (28), since a rat vibrissa grows at least 1–1.5 mm/day for the 4 weeks of the anagen phase (24).

Our data explain the observations that regeneration of whiskers can occur in follicles in which no more than the lower one-third of the follicle is removed (i.e., the matrix area and the dermal papilla) (15, 29). They provide experimental verification for the hypothesis that the follicular stem cells that are the reservoir of keratinocytes responsible for the long-term growth of the hair reside in the hair bulge (13). The colony-forming ability and the [^{3}H]thymidine-retaining ability of a cell appear to be the expression of a unique phenotype linked to the stem cell or its close progeny.

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