Int. J. Dev. Biol. 36: 373-380 (1992)

Original Article

Hydrocortisone perturbs the cell proliferation pattern during feather morphogenesis: evidence for disturbance of cephalocaudal orientation

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ABSTRACT In this study, we have monitored the spatial distribution of S-phase cells during successive stages of normal feather morphogenesis using the specific marker BrdU. We also disturbed the developmental program by administration of hydrocortisone on the chorioallantoic membrane of 6.5-day chick embryos and examined the resulting pattern of BrdU incorporation. Our results show that a specific spatio-temporal pattern of cell proliferation occurs during successive stages of feather development and that this pattern accounts for the growth of feather buds according to the cephalocaudal orientation. Our experimental analysis showed that the stage-dependent alteration of feather morphogenesis (as shown by Züst, *Ann. Embryol. Morphogen. 4,* 1971 and confirmed by Démarchez *et al., Dev. Biol. 106,* 1984), is based on a stage-dependent alteration of the proliferation pattern in the epidermis. Forty-eight hours after treatment, non-induced epidermis ceases DNA synthesis and is unable to form placodes. Induced epidermis at the placodal and dermal condensation stages fails to produce the cohorts of S-phase cells responsible for the caudal outgrowth and the slanting shape of the buds. These young buds display anarchic proliferation in the whole epidermis possibly resulting in the appearance of «curly» feathers. Together, these results show the importance of the spatial pattern of ectodermal and mesodermal cell proliferation during the normal feather morphogenesis. Moreover, they corroborate the particular role of epidermis both in the establishment of feather rudiments and in the cephalocaudal orientation of the feathers.

KEY WORDS: *feather morphogenesis, proliferation, hydrocortisone, cephalocaudal axis*

Introduction

Epithelial-mesenchymal interactions occurring during feather morphogenesis result in the proliferation, migration and differentiation of cell populations according to a specific spatio-temporal pattern. In a previous work (Desbiens *et al.*, 1991), we have described the spatial distribution of S-phase cells by monitoring the incorporation of 5-bromo-2-deoxyuridine (BrdU) during successive stages of skin morphogenesis. In order to assess a putative relationship between cell proliferation and appendage building, we have considered the possibility of disturbing the normal developmental program in the skin. In agreement with previous studies (Züst, 1971), Démarchez *et al.* (1984) have shown that injection of 0.1 mg hydrocortisone in the yolk sac of 6-day chick embryos led to nearly total absence of feathers over the whole pterylae. The same treatment performed at 6.5-day allowed the development of one to three rows of feathers along the mid-dorsal area. It was observed that a general decrease of cell proliferation in the dermis of glabrous zones correlated with an increase in interstitial collagen synthesis and deposit. However, the spatial pattern and temporal sequence according to which cells proliferate was not described in these experimental conditions. The aim of the present study was to investigate the role of both ectodermal and mesodermal cell proliferation in the process of normal feather development and in hydrocortisone-disturbed morphogenesis. Here we show that hydrocortisone-treated embryos display an overall decrease in proliferation and a conspicuous alteration of spatial distribution of S-phase cells both in the epidermis and the dermis leading to lateral glabrous areas on the sides of mid-dorsal «curly» feathers. We suggest that coordination of epidermal and dermal cell proliferation explains the spatial pattern of feather morphogenesis, and the oriented outgrowth of the feather buds when the appendages emerge and slant backwards to elongate in a cephalocaudal direction.

Abbreviations used in this paper: BrdU, 5-bromo-2-deoxy-uridine.

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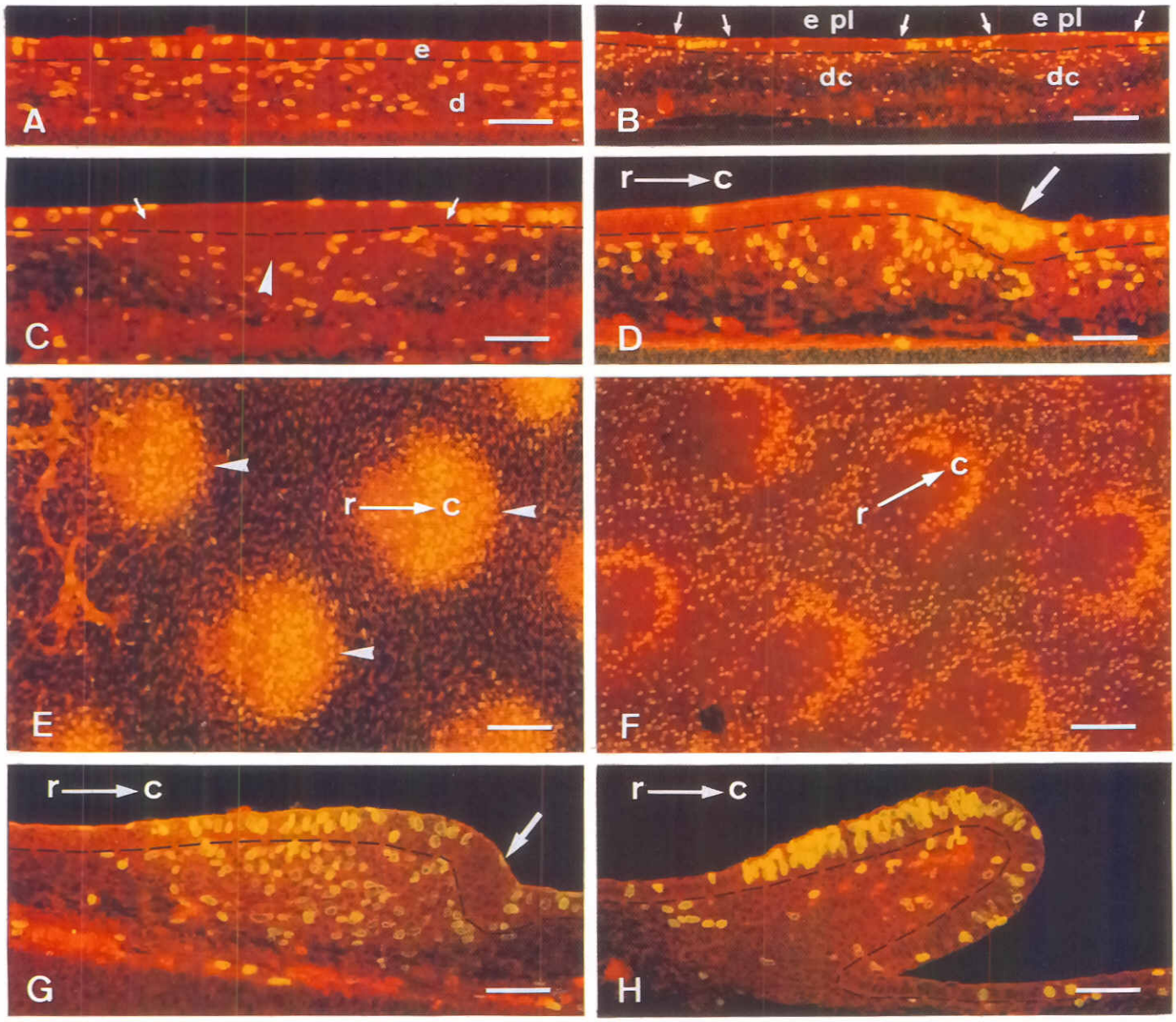


Fig. 1. Detection of DNA-synthesizing cells during normal feather bud formation. *The dermal-epidermal junction is indicated by a broken line. At the completion of the pre-dermal cell accumulation* **(A)**, *numerous labeled nuclei can be seen randomly distributed both in the epidermis (e) and in the dermis (d). Next,* **(B)** *dermal cells migrate and aggregate under unlabeled epidermal placodes (e pl). Epidermal interplumar areas are strongly labeled (arrows), dermal condensations (dc) display numerous labeled nuclei. At the completion of dermal condensation* **(C)**, *sagittal sections show that both the placodal cells (arrows) and the dermal cells situated in the core of the aggregate (arrow head) fail to incorporate BrdU. However, some peridermal cell nuclei and peripheral dermal cell nuclei are labeled. The outgrowth of the caudal edge of the feather rudiment* **(D)** *is associated with a local increase in proliferation as seen in sagittal sections. In the dermis, a section parallel to the skin surface* **(E)** *shows more numerous labeled nuclei in the caudal half of each rudiment (arrow head) than in its cephalic half, while whole-mounts of epidermis* **(F)** *show labeled cells chiefly grouped in crescents at the caudal edge of the otherwise unlabeled epidermal placodes. As the rudiments emerge from the back skin, sagittal sections show the opposite pattern of labeling: the S-phase cells are predominantly seen over the whole cephalic epidermal sheet while the caudal edge does not contain proliferative cells* (**G**, *arrow). As the feather bud slants backward* **(H)**, *numerous labeled cells can be seen in the epidermal sheath, while labeled nuclei are sparse in the dermal pulp. r → c rostral to caudal orientation. Bars: 50 μm for A, C, D, G, H. Bars: 100 μm for B, E, F.*

Results

Normal development

Until E 5 (E 0 being the first day of incubation), the future epidermis displays a basal layer of columnar cells under a peridermal layer of flattened cells. The underlying mesoderm is formed by loose pre-dermal cells. BrdU-incorporating nuclei were scattered both in the epidermis and in the loose pre-dermis. As the accumulation of mesenchymal cells underneath the epidermis began, numerous cell nuclei incorporated the thymidine analog. The number of

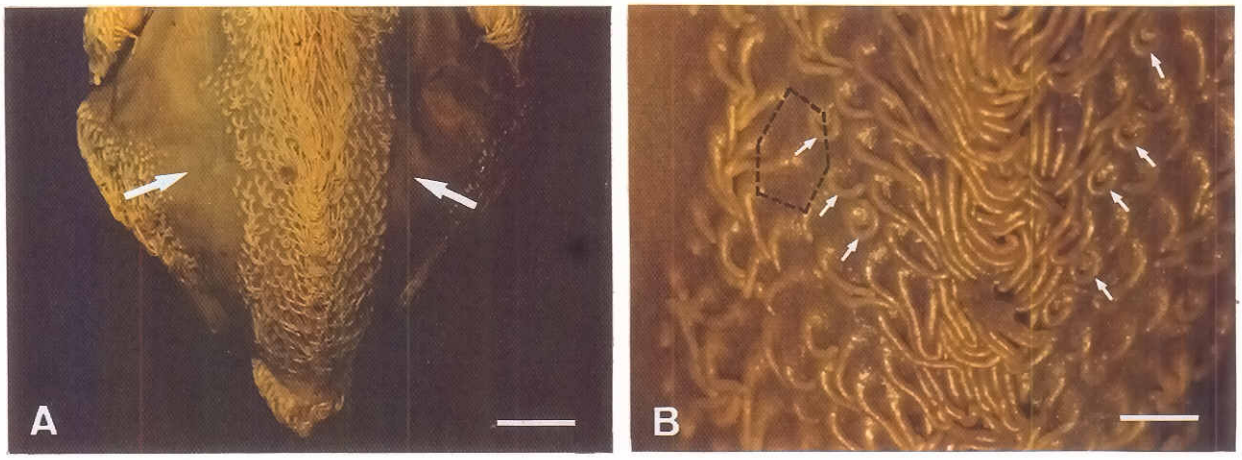


Fig. 2. Macroscopic effects of the hydrocortisone treatment at E 6.5. (A) *Two glabrous areas can be seen on each side of the narrow mid-dorsal pteryla (arrows) of a treated embryo examined at E 12.* **(B)** *An enlarged view shows that feather buds are arranged in a normal hexagonal pattern (broken line), but that numerous rudiments do not display the correct cephalocaudal orientation but rather display a curly appearance (arrows). Bar: 2 mm for A. Bar: 0.5 mm for B.*

labeled nuclei also increased in the epidermis without any particular spatial organization (Fig. 1A).

When the first rows of circular epidermal placodes appeared along the mid-dorsum at E 6, a typical pattern of BrdU incorporation was observed in whole-mounts of epidermis. Circular placodes almost devoid of labeling were surrounded by numerous S-phase cells in the interplumar areas (Fig. 3A-B). Then, as shown by Sengel and Rusaouën (1968), dermal cells migrated along a network of interstitial collagenous fibers (see Dhouailly, 1984, Fig. 7) to form local dermal condensations beneath each epidermal placode. Sagittal sections of feather rudiments showed numerous aggregating cells incorporating BrdU (Fig. 1B). Later, at the completion of the dermal condensation, aggregated cells located in the center of the dermal condensations as well as cells within the placodes failed to incorporate BrdU (Fig. 1C and Desbiens *et al.*, 1991 Fig.4). Placodal labeled cells observed in whole-mounts were situated in the peridermal layer.

At the onset of the feather outgrowth (E 7-E 8), the number of S-phase cells increased in the caudal part of the feather rudiment dermis (Fig. 1D-E). On the other hand, the caudal edge of the epidermal placodes also displayed numerous labeled nuclei whereas the cephalic edge was unlabeled (Fig. 1D). Epidermis whole-mounts showed fluorescent crescents delineating the caudal edge of each rising bud and an overall decrease of labeling in the previously highly proliferative interplumar areas (Fig. 1E).

As the buds emerged from the skin (E 8.5), dermal cells incorporated BrdU. Moreover, the part of the epidermal sheet that previously did not contain S-phase cells displayed a strong labeling suggesting that numerous quiescent placodal cells had re-entered the cell cycle at this time. Conversely, those epidermal S-phase cells that formed the caudal edge of the rising bud ceased to incorporate BrdU (Fig. 1G). Whole-mounts of epidermis corroborated these observations with a reverse pattern of BrdU incorporation when compared to the previous step of morphogenesis. Interplumar areas contained sparse labeled nuclei while the epidermal covering of each bud showed a conspicuous labeling that progressively decreased from the caudal to the cephalic edge of the feather rudiment (Fig. 3G).

As the buds elongated and slanted backwards, BrdU incorporation was essentially localized in epidermal cell nuclei. However, the number of labeled nuclei was higher in the dorsal part of young epidermal sheaths than in their ventral part (Fig. 1H). On the other hand, the number of S-phase dermal cells drastically decreased in the core of the bud.

Effects of hydrocortisone treatment on skin development

Macroscopic observations

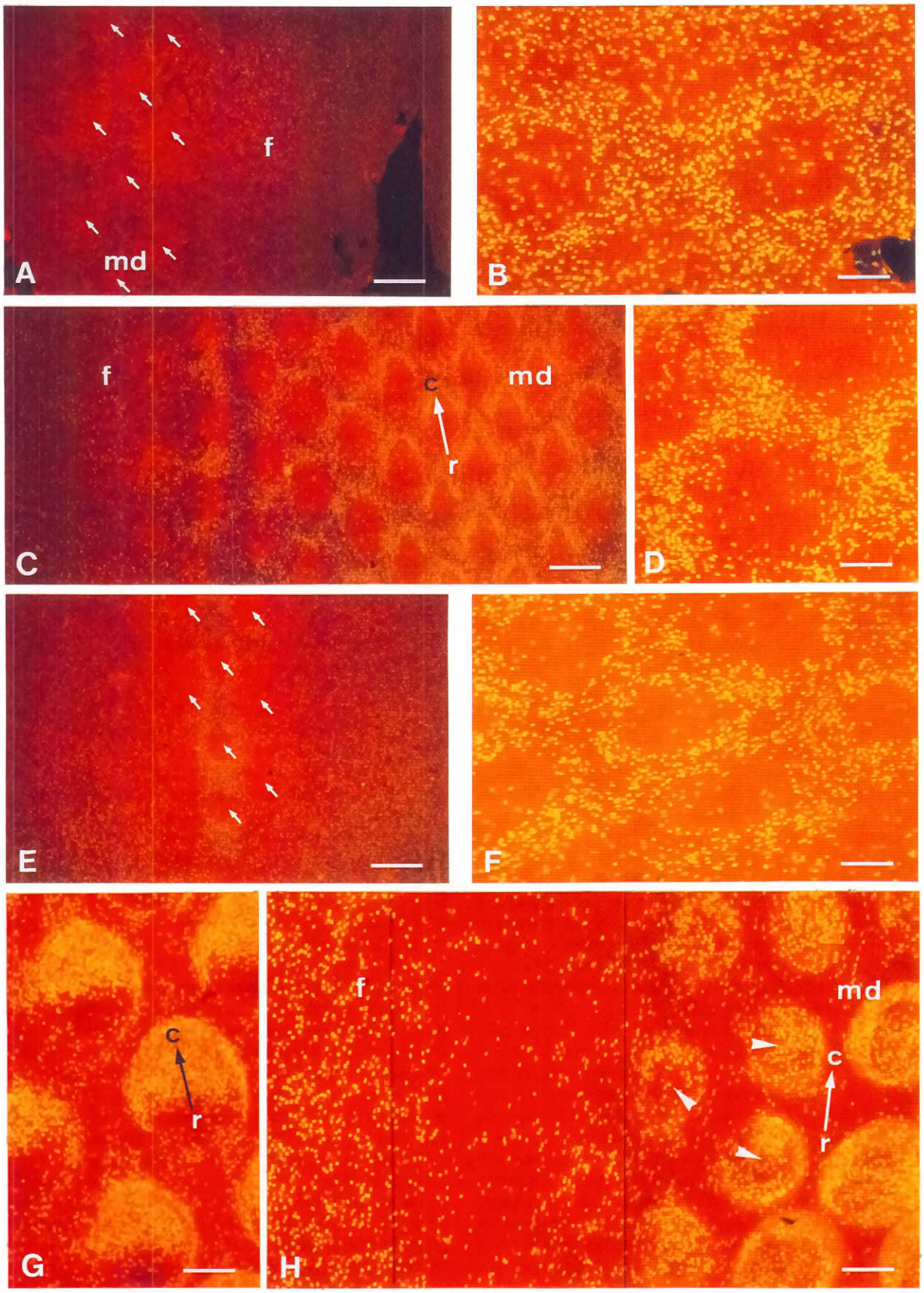
In our hands, the injection of 0.1 mg hydrocortisone at E 6.5 allowed the development of several feather rows along the mid-dorsum of embryos while on both sides the skin remained glabrous (Fig. 2A). As noticed by Démarchez *et al.* (1984), the width of the feathered area and consequently the number of feather rows depended upon the precise time when drug treatment was performed. The feathers located at the edge of the lateral glabrous areas were not elongated in a cephalocaudal direction but rather presented a «curly» appearance (Fig. 2B). However the hexagonal feather pattern did not seem to be affected (Fig. 2B). When several feather rows appeared, the mid-dorsal feathers elongated according to the normal cephalocaudal orientation.

Effect of treatment on BrdU incorporation

Control explants excised at the time of addition of hydrocortisone (E 6.5) displayed typical axial placodes devoid of labeling with underlying dermal condensations (Fig. 4A). Laterally, on both sides, the skin displayed numerous labeled nuclei randomly distributed both in the dermis and the epidermis (Fig. 4B). Whole-mounts of epidermis corroborated the presence of some rows of unlabeled placodes along the mid-dorsal area of the back skin (Fig. 3A).

During normal development longitudinal rows of feather buds arise sequentially in a continuous process on both sides of the initial mid-dorsal row. Whole-mounts of epidermis from control embryos at E 7.5 revealed a gradient of development from the protruding axial feather germs to flat and uniform epidermis in the ventral regions (Figs. 3C-D).

At E 7.5, 24 h after hydrocortisone treatment, the back skin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seemed to have stopped morphogenesis. Compared to E 6.5 controls, no additional rows of placodes were seen in whole-mounts of epidermis (Fig. 3E), and the number of BrdU-incorporating nuclei decreased in the interplumar areas (compare Fig. 3F and Fig. 3B-D). Serial longitudinal sections showed that more developed rudiments displayed features of E 6.5 feather buds, i.e., unlabeled epidermal placodes and labeled aggregating dermal cells (Fig. 4C). Both low and high magnifications corroborated the decreased number of labeled cell nuclei in interplumar epidermis (Figs. 4C-D). The skin at the edge of these feather rudiments seemed to incorporate BrdU as did control flank skin (Fig. 4E).

At E 8.5, 48 h after hydrocortisone treatment, whole-mounts of epidermis showed some axial asymmetric sheaths similar to controls while more laterally circular feather sheaths comprising more uniformly labeled nuclei were observed (Fig. 3H). In some cases, an abnormal transverse gradient of labeled epidermal nuclei was seen (Fig. 3H). This distribution of S-phase cells contrasted with the asymmetric outgrowth and polarized distribution of labeled nuclei observed in control specimens either at E 7.5 or E 8.5. On the other hand, more laterally, we never observed the typical unlabeled epidermal placodes surrounded by highly proliferating interplumar cells. Instead, between disturbed feather germs and the flanking region appeared a strip of skin where only sparse nuclei incorporated BrdU as in the mid-dorsal interplumar areas (Fig. 3H). Transverse and sagittal sections were performed in order to gain some insight into this peculiar development. *Serial longitudinal sections* showed small slanting buds in the mid-dorsal area of the more developed embryos. Irrespective to their reduced size, the pattern of S-phase cell distribution in these buds was identical to that of untreated slanting buds. However, the number of labeled nuclei was reduced (Fig. 4F). More laterally, small symmetric feather germs comprising a reduced number of labeled dermal cells were seen. The normal asymmetric distribution of these dermal cells relative to the cephalocaudal axis disappeared (Fig. 4G). In most cases, labeled cells were observed in the whole epidermal sheath of each protruding bud (Fig. 4H). *Transverse sections* revealed asymmetric accumulations of epidermal S-phase cells from emerged buds as already suggested by whole-mounts of epidermis (Fig. 4I).

Lateral «glabrous» areas were characterized by unlabeled epidermal cells whereas proliferation was observed among underlying dermal cells (Fig. 4J).

Longitudinal sections performed 3 days after treatment at the edge of the glabrous area showed rudiments growing perpendicularly to the skin plane and others which were accurately slanted (data not shown).

Discussion

In this report we have mapped the spatial distribution of S-phase cells by monitoring the incorporation of BrdU during successive steps of both normal and hydrocortisone-disturbed development of feather rudiments. In normal morphogenesis, we showed that each stage is characterized by a particular pattern of S-phase cell distribution.

The accumulation of dermal cells

In the dorso-lumbar area, dermal cells arise from the dermatomal part of somites. They leave the dermatome and colonize the subepidermal space as early as E 3 (Sengel, 1970). Thus the increase in dermal cell density has been ascribed to an increased proliferation of the cells starting at E 5-E 6 rather than to a late invasion of dermal stem cells. Indeed, we and others previously showed an increased proliferation in the feather-forming regions of embryos between late E 5 and E 6 (Wessells, 1965; Desbiens *et al.*, 1991). Wessells (1965) has estimated that the dense dermis contained 2.6 nuclei/1000 μm^3 while the subcutaneous mesenchyme contained 1.9 nuclei/1000 μm^3 at the end of this process. Proliferating cells were randomly distributed both in the epidermis and the dermis so that no prediction could be drawn concerning subsequent morphogenetic events.

The epidermal placodes and dermal condensations formation

The newly established epidermal placodes ceased DNA synthesis while surrounding interplumar epidermal cells uniformly incorporated BrdU. Likewise, as dermal cells reached the center of the dermal condensation, they failed to incorporate BrdU whereas peripheral and interplumar cells continued to incorporate BrdU. Histological investigations (Sengel, 1970) showed that the density of epidermal cells in the placodes did not increase corroborating the lack of proliferative activity. On the other hand, Sengel and Rusaouën (1968), Sengel (1970), Stuart *et al.* (1972) showed that interplumar mesenchymal cells migrated along pre-established axes between the feather rudiments and aggregated beneath the placodes. Two major populations of cells were seen in the condensations: quiescent cells in the center and highly proliferative cells at the periphery (Wessells, 1965; Sengel, 1970). Our experiments agree with this description and account for a dual origin of dermal condensations. Peripheral cells aggregate after a centripetal migration and divide to reach in the condensation core a critical density possibly inconsistent with further proliferation (5.5 nuclei/1000 μm^3 according to Wessells, 1965).

Fig. 3. Detection of DNA synthesizing cells in whole-mounts of epidermis from control and treated embryos. *Control epidermis at E 6.5* **(A-B)** *shows some rows of unlabeled placodes (arrows) surrounded by numerous labeled interplumar nuclei in the mid-dorsal area (md). S-phase cells are numerous and randomly distributed in the flank area (f). Control epidermis at E 7.5* **(C)** *displays a gradient of development from the axial protruding feathers to neighboring unlabeled placodes and more laterally uniformly labeled flank cells. Unlabeled placodal cells are surrounded by numerous labeled nuclei* **(D)**. *24 h after the hydrocortisone treatment, whole-mounts of epidermis show general features similar to those of controls at E 6.5* **(E)**. *No additional rows of feather rudiments appear; moreover epidermal placodes seem to stop their development. A higher magnification* **(F)** *shows a slight decrease of BrdU incorporation in the interplumar cells when compared to controls (B and D). 8.5-day control embryos* **(G)** *show axial feathers whose epidermal sheaths strongly incorporate BrdU. One can observe a conspicuous rostrocaudal gradient of labeling contrasting with the sparsely labeled interplumar areas. 48 h after the treatment, whole-mounts of epidermis display atypical feather rudiments* **(H)**. *Some of them show circular uniformly labeled sheaths while others show accumulation of S-phase cells towards lateral edges of the buds (arrow heads). These S-phase cell distributions do not agree with the specific cephalocaudal pattern of proliferation observed in control embryos. Laterally, characteristic unlabeled placodes are not seen. Instead, a poorly labeled epidermis is located between atypical feathers and the highly proliferative flank epidermis. r → c: rostral to caudal orientation. md: mid-dorsal area. f: flank region. Bars: 250 μm for A, C, E. Bars: 100 μm for B, D, F, G, H.*

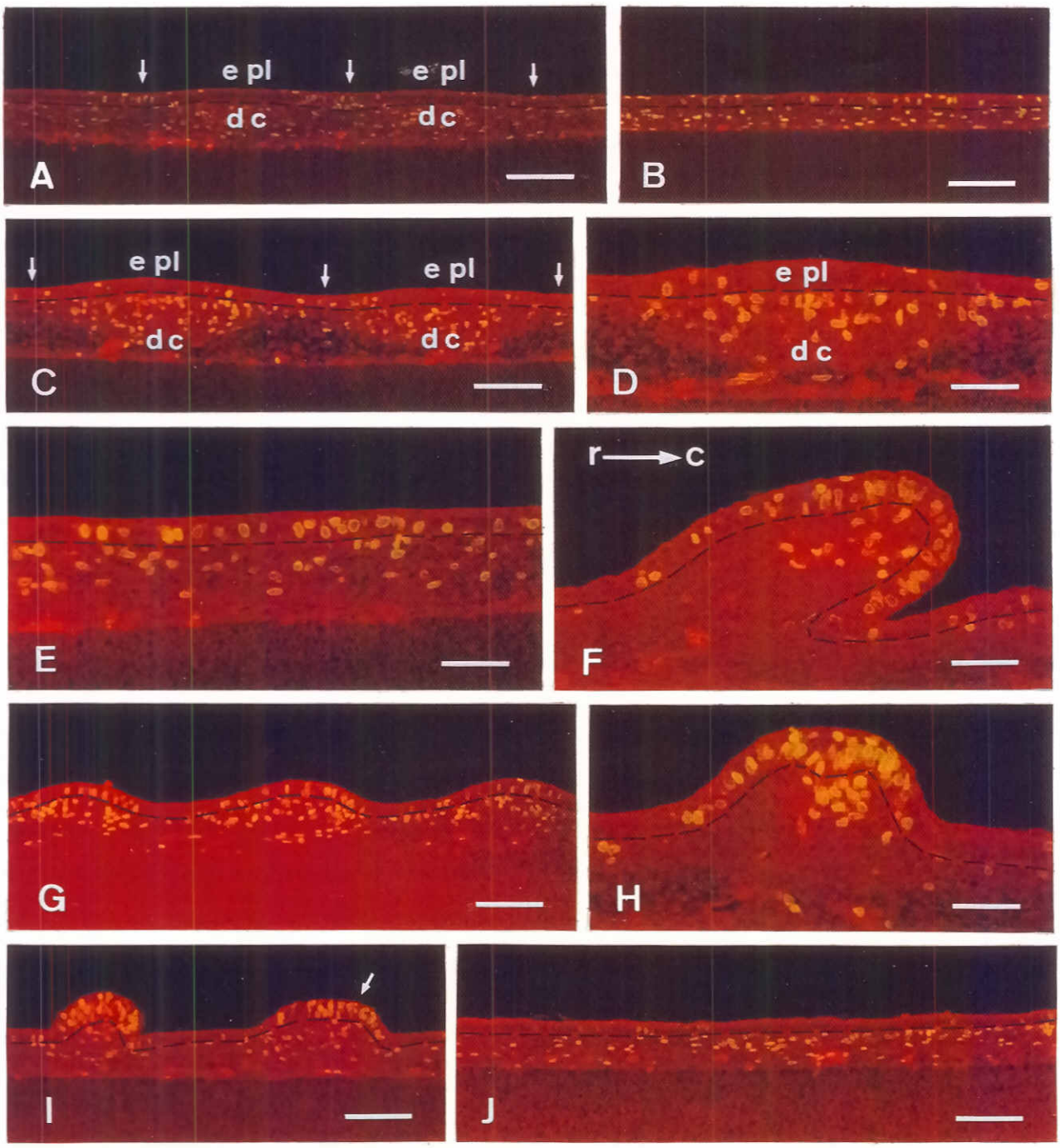

Fig. 4. Detection of DNA synthesizing cells in sagittal and transverse sections of hydrocortisone-treated skin. *Sagittal sections of control explants at the time of treatment* **(A)** *show typical unlabeled placodal cells (e pl) surrounded by interplumar labeled cells (arrows) and associated dermal condensations (dc) with their peripheral labeling. More laterally, S-phase cells are randomly distributed both in the epidermis and the dermis* **(B).** *24 h after the hydrocortisone treatment* **(C,D)**, *sagittal sections show feather rudiments comprising unlabeled placodal cells (e pl) and uniformly labeled dermal condensations (dc). It is noteworthy that interplumar areas fail to incorporate BrdU contrary to controls and that no gradient of labeling can be detected in the rostrocaudal axis of the feather buds (compare to Figs. 1D and 3C). Flank skin incorporates BrdU in both epidermis and dermis* **(E)**. *48 h after the treatment, midsagittal sections of more developed feather buds are similar to controls; however the number of S-phase cells appears reduced in the epidermal sheath* **(F)**. *More laterally, atypical outgrowth occurs: buds emerge without cephalocaudal polarity* **(G)**. *S-phase cells are randomly distributed both in the epidermis and in the dermis. Numerous feather germs display a uniform symmetric labeled epidermis relative to the cephalocaudal axis* **(H)**. *When transverse sections are observed* **(I)** *asymmetric distribution of incorporating nuclei (arrow) accounts for a transverse growth of the germs. Longitudinal sections of lateral skin* **(J)** *show that the epidermis does not incorporate BrdU while the dermis displays numerous S-phase cells. r → c: rostral to caudal orientation. Bars: 100 μm for A, B, C, G, I, J. Bars: 50 μm for D, E, F, H.*

The outgrowth of feather buds

24 h after the appearance of the first epidermal placodes, the distribution of S-phase cell populations was quite altered. We observed labeled cells towards the caudal edge of the feather bud both in the epidermis and the dermis. This increased BrdU incorporation in the caudal edge of each rudiment agrees with the hypothesis of a local outgrowth accounting for the asymmetric shape of the young feather germ. The presence of epidermal cell proliferation towards the caudal edge of the feather buds suggests that the epidermal placodes are not simply pushed up by the enhanced proliferation of underlying dermal cells but rather cooperate with this cell population to raise the caudal part of each bud.

Later, whole-mounts as well as sagittal sections showed numerous labeled nuclei within the dorsal part of the feather sheath while cells situated at the caudal edge of the bud displayed resumed proliferation. A gradient of label was also observed decreasing from the caudal to the cephalic edge of the epidermal sheath. This pattern could explain an elongation of the dorsal epidermis between the two putative anchorage zones of unlabeled cells. Experiments of pulse labeling with increasing times of chase could gain some insight into the hypothesis of a slippage towards the caudal edge of the bud according to a mechanism described by Tanaka and Kato (1983) during the morphogenesis of avian scales. The overall increase of DNA synthesizing cells in the dermis accounts for the outgrowth of the whole feather germ when one considers the drastic decrease of proliferation in the interplumar areas (both in the epidermis and the dermis).

As the feather buds slanted backwards and elongated in the caudal direction, the plumar epidermis strongly incorporated BrdU first in the dorsal part possibly to achieve the caudal slant of the feather and the rising of the first barb ridges. Then the whole sheath incorporated BrdU to ensure the elongation of the feather. Conversely, the density of labeled nuclei in the dermis decreased. Our unpublished observations showed an overall decreased density of dermal cells that were accumulating great amounts of extracellular matrix components from the proteoglycan family (see also Jahoda *et al.*, 1987).

The effects of the hydrocortisone treatment on proliferation at the onset of skin morphogenesis first appeared as an inhibition of the establishment of the normal proliferation pattern. Indeed, 24 h after the treatment (E 7.5), experimental embryos displayed specific features of E 6.5 embryos: new rows of unlabeled placodes were not seen. In the same way, whole-mounts of dermis (data not shown) did not reveal additional rows of condensations. These results agree with data reported by Démarchez *et al.* (1984) who did not see morphological changes in the skin of embryos 24 h after the treatment except an increased amount of collagen in the dermis. These authors concluded that «during the first 24 h, hydrocortisone appears to slow down or inhibit morphogenesis while it stimulates biochemical maturation of the dermis».

The observation of dorsal skin of embryos 48 h after hydrocortisone treatment suggests that the response of each feather rudiment row depends upon its physiological state when hydrocortisone reaches the pteryla. Mid-dorsal rows seem to have overcome a critical state of sensitivity and develop a harmonious cephalocaudal polarity. Younger lateral rows are highly susceptible to hydrocortisone effects since they grow more slowly, in some cases perpendicular to the skin plane, and their orientation is disturbed (curly appearance). Non-induced lateral epidermis becomes unable to initiate morphogenesis and gives rise to glabrous areas with a total inhibition of additional epidermal placode formation. Since we never saw cohorts of S-phase cells spatially localized at the caudal edge of treated feather rudiments, it can be postulated that this early step is inhibited by the hydrocortisone treatment. A possible association between anarchic proliferation in epidermis and the «curly» aspect of feather filaments appears relevant according to the previous demonstration that the cephalocaudal orientation of skin appendages is epidermis dependent (Sengel, 1957).

Lateral areas that included still numerous labeled nuclei 24 h after treatment both in the epidermis and the dermis subsequently ceased DNA synthesizing activity only in the epidermis. This observation further substantiates the proposal of Démarchez *et al.* (1984) that epidermis loses its appendage-forming ability and undergoes a precocious differentiation. Secondarily, the dermis differentiates and accumulates interstitial collagens. Histological analysis performed by Stuart *et al.* (1972) on hydrocortisone-treated skins showed that the epidermis was thicker than normal and that dermal cells were smaller and more closely packed. Experimental recombination of treated epidermis with untreated dermis cannot produce feathers, while the reverse association of untreated epidermis with treated dermis can (Stuart *et al.*, 1972). These experiments and our observation that the distribution pattern of curly feathers was not disturbed show that hydrocortisone treatments do not seem to inhibit the multistep control exerted by dermis upon epidermis, but that treated epidermis is unable to respond to one (or more?) of these stimuli (see Sengel, 1990 for a detailed review). These problems remain to be solved by further analysis.

In conclusion, in the present study, we have performed a temporal and spatial analysis of hydrocortisone effects on proliferation in the two compartments of the skin during feather morphogenesis. It should be recalled that normal development requires the proliferation of cell cohorts from both epidermal and dermal lineages in complement to changes in extracellular matrix or cell-adhesion molecule synthesis and deposition (Mauger *et al.*, 1982; Chuong and Edelman, 1985; Gallin *et al.*, 1986; Jahoda *et al.*, 1987; Kitamura, 1987; Goetinck and Carlone, 1988; Tucker, 1991). Experimental analysis using hydrocortisone treatment shows the importance of the cell cycle regulation in epidermal cells during feather pattern establishment and subsequent morphogenesis.

Materials and Methods

Biological model

As recommended by Démarchez *et al.* (1984), eggs from the white Sussex strain were incubated at 38.5°C until they reached E 6.5 (E 0 being the first day of incubation). In order to compare feather forming and featherless skin within the same treated specimen, control or treated embryos received 0.1 ml of PBS or 0.1 mg hydrocortisone (hydrocortisone 21 sodium succinate; Sigma) in 0.1 ml of PBS respectively. The drug was deposited on the chorioallantoic membrane at the level of the vascular area edge. Dorso-lumbar skin was pealed off the back of embryos at E 6.5 for controls before treatment, at E 7.5 to E 9.5 for control and treated skin (stages 29 to 36 according to Hamburger and Hamilton, 1951).

Detection of DNA synthesizing cells

BrdU incorporation

Freshly excised rectangular pieces of skin (epidermal side facing up) were cultured on filtration membranes (1.22 μm pore size, Millipore) carried by grids of stainless steel placed in 35 mm Petri dishes. The level of medium was adjusted so as to soak the filters. The medium used in this study has

been described by Markwald *et al.* (1990): it comprises Medium 199 (Gibco) supplemented by ITS (Sigma) i.e., insulin (5 μg ml^{-1}), transferrin (5 μg ml^{-1}), selenium (5 ng ml^{-1}), 1% chick serum (Gibco), antibiotics (penicillin: 50 iu ml^{-1}, streptomycin: 50 μg ml^{-1}), and 50 μM BrdU (Sigma). Culture dishes were incubated at 37°C in a damp atmosphere of 5% CO_2 in air. Skin pieces were fixed in Bouin's fluid 3 h after the beginning of incubation.

Histological and whole-mount procedures

Whole-mount specimens of skin epidermis at E 6.5, E 7.5 and E 8.5 were prepared as previously described (Desbiens *et al.*, 1991). Briefly, cultured explants spread over Millipore filter with epidermal side facing down were fixed for 15 min in Bouin's fluid, extensively washed in PBS and immersed in a 0.2% solution of EDTA in Ca^{2+}- and Mg^{2+}-free PBS overnight at 37°C. The dermis was discarded using fine forceps. The epidermal layer was then freed from the filter, fixed for 2 additional hours and processed *in toto* for the detection of BrdU-labeled nuclei as described in the following section. In other cases, cultured specimens were fixed in Bouin's fluid, washed and embedded in paraffin. Embedded explants were cut nominally 7 μm thick either longitudinally, parallel to the cephalocaudal and dorsoventral axis of the embryo, transversely to the cephalocaudal axis or parallel to the skin surface.

Immunological procedures

Isolated epidermis or deparaffinized sections were treated according to the anti BrdU-DNA monoclonal antibody manufacturer instructions (Partec). Denaturation of DNA was performed by incubating specimens in 1.5 N HCl for at least 20 min at room temperature. After 2 washes in PBS, specimens were incubated in a 1/100 dilution of the anti BrdU-DNA antibody in PBS pH 7.4 supplemented with 0.5% Tween 20 and 0.5% bovine serum albumin (Sigma). Sections were incubated for 1 h, but whole-mounted epidermis required 2 additional hours to achieve complete binding of the antibody to its target. Specimens were washed in PBS and immersed in a 1/100 dilution of fluorescein-conjugated rabbit anti-mouse IgG (Sigma) in PBS pH 7.4 supplemented with 0.5% Tween 20 and 1% neutral goat serum for 1 to 2.5 h according to the type of specimen. After linkage of the FITC conjugate, a wash in PBS was performed and the preparations were stained with Evans blue (1/10,000). Epidermis or sections were mounted in glycerol/PBS. Controls were performed where the primary antibody was replaced by PBS. Specimens were examined and photographed using an Olympus BH2 epifluorescence photomicroscope.

Acknowledgments

We thank Drs. P. Sengel, A. Bart, A. Capuron and C. Quéva for critical reading of the manuscript and for valuable discussions, F. Laloux for patient typing. This work was supported by grants from the Association pour la Recherche sur le Cancer.

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Accepted for publication: June 1992