Programming gene expression in developing epidermis

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SUMMARY

As the major proteins of adult keratinocytes, keratins provide biochemical markers for exploring mouse epidermal embryogenesis. Here, we used a modified method of whole-mount in situ hybridization to track skin-specific expression of endogenous keratin mRNAs throughout embryogenesis. To monitor transcriptional regulation, we coupled this with β-galactosidase expression of a human epidermal keratin promoter-driven transgene. These studies have radically changed our perception of how the program of gene expression becomes established during epidermal development. Specifically, we have discovered that (1) basal keratin (K5 and K14) genes are first detected at E9.5 in a highly regional fashion, and surprisingly as early as the single layered ectodermal stage; (2) the early patterns do not correlate with morphogenesis per se, but rather with regional variations in the embryonic origin of underlying mesenchyme, supporting morphogenetic criteria that early inductive cues are mesenchymal; (3) epidermal keratin genes are expressed in periderm, supporting the notion that this layer arises from ectodermal stratification, even though it is simple epithelial-like in morphology and is subsequently sloughed during development; (4) later embryonic patterns of K5 and K14 gene expression parallel proliferative capacity and not stratification; and (5) K1 and K10 mRNAs are first detected as early as E13.5, and their patterns correlate with differentiation and not stratification.

These patterns of epidermal gene expression led us to explore whether potential transcriptional regulators of these genes are expressed similarly. We show that AP2 (but not Sp1) cRNAs hybridize in a pattern similar to, but preceding that of basal keratin cRNAs. Finally, using gene expression in cultured cells, we demonstrate that AP2 has a strong inductive effect on basal keratin expression in a cellular environment that does not normally possess AP2 activity.

Key words: embryonic ectoderm, keratins, loricrin, AP2, gene expression, skin

INTRODUCTION

Adult epidermis is a stratified epithelium in which cells in the innermost, basal layer cease to divide concomitantly with a process of upward movement and terminal differentiation. Dead squames sloughed from the skin surface are proteaceous, cross-linked envelopes, or sacs, filled with highly bundled, macrofibrils of hundreds of keratin filaments. The biochemical program of terminal differentiation involves the sequential expression of different proteins, including keratins in the basal and spinous layers, and loricrin, a component of the cornified envelope, in the granular layer.

Type I (K9-K20) and type II (K1-K8) keratins form obligate heterodimers that constitute the subunits of epithelial intermediate filaments (IFs) (reviewed by Fuchs and Weber, 1994). Simple epithelia express K8 and K18 (Wu and Rheinwald, 1982), while basal cells of adult epidermis express K5 and K14 (Fuchs and Green, 1980; Nelson and Sun, 1983). As epidermal cells become committed to terminal differentiation, they switch from expression of K5 and K14 to K1 and K10 (Fuchs and Green, 1980; Roop et al., 1987). Hair follicles display more elaborate programs of differentiation and keratin expression, including K5 and K14 in the outer root sheath (ORS), K1 and K10 in the inner root sheath (IRS) and hair-specific keratins in the cortex and medulla (Heid et al., 1986; Lynch et al., 1986; Stark et al., 1990).

Much of what is known about embryonic epidermal gene expression in mammals comes from characterization of skin proteins, and from immunohistochemistry of skin sections, often stained with antibodies of multiple keratin specificities. Some studies have suggested that early in development, when the epidermis is only a single layer, cells express simple epithelial keratins (Jackson et al., 1981; Franke et al., 1981). While subsequent studies with monoclonal antibodies have not detected K8 and K18 at very early stages (E7.5), there is agreement that K8 and K18 localize to the flattened upper layer of periderm cells (Moll et al., 1982; Dale et al., 1985). Intriguingly, periderm also has desmosomes and can express involucrin, suggesting certain similarities to epidermal as well as simple epithelial
cells (Watt et al., 1989). In later stages of development, periderm is apparently sloughed (Sengel, 1976), making its origin even more difficult to assess.

Expression of K5 and K14 has been detected as early as the bi-layered epithelial stage (Moll et al., 1982; Schweizer and Winter, 1982; Banks-Schlegel, 1982; Dale et al., 1985; Fisher and Holbrook, 1987). Immunohistochemistry using a mono-specific anti-K14 antiserum and in situ hybridization with a K14 cRNA probe has localized expression to the inner, embryonic basal layer of back skin sections (Kopan anduchs, 1989). After stratification, K14 seemed to be restricted to the innermost layer, in agreement with earlier studies using less specific probes. When combined with protein synthesis data and morphological correlations, these findings have led to the assumption that K5 and K14 genes are downregulated upon stratification. Researchers have uniformly stressed the intimate correlation between these keratins and a single layer of columnar, basal cells.

It is well-accepted that K1 and K10 induction occurs post K5 and K14 (Moll et al., 1982; Banks-Schlegel, 1982; Schweizer and Winter, 1982; Dale et al., 1985; Kopan and Fuchs, 1989), and times as early as E15.5 have been reported (Greer and Roop, 1991). K1 and K10 have often been detected concomitantly with the appearance of a third, middle epidermal layer, suggesting a link between these keratins and stratification (Moll et al., 1982; Schweizer and Winter, 1982; Dale et al., 1985; Kopan and Fuchs, 1989). Collectively, keratin studies have led investigators to conclude that there are strong parallels between embryonic and adult epidermis, with K5 and K14 gene expression restricted to the inner layer, and K1 and K10 confined to stratifying layers.

Despite the relative consistency of these findings, we found their logical predictions difficult to reconcile in light of earlier morphological studies and tissue reconstitution experiments. For many years, developmental biologists have underscored the importance of underlying mesenchyme in epidermal embryogenesis, emphasizing that mesenchyme is highly regional and stems from different origins (for reviews, see Sengel, 1976, 1990; Hardy, 1992). Mesenchymal influence has been implicated in proliferation, maintenance and differentiation of embryonic epidermis and its appendages (Wessels, 1963; Noden, 1988; Richman and Tickle, 1989; reviewed by Sengel, 1990). Differences in underlying mesenchyme also seem to account for certain regional morphological heterogeneities in ectoderm (Hanson, 1947; Sengel, 1976). This said, epidermal morphologies are sometimes difficult to distinguish even when cells overlie different mesenchymes and will develop differently. Such findings have led us to wonder whether K5 and K14 expression in early development strictly correlates with a single layer of columnar basal cells, or whether environmental cues might also be involved.

Another distinguishing aspect of embryonic epidermis is that upon stratification, suprabasal cells often retain basal-like morphology and mitotic activity. This feature may satisfy the need for rapid surface expansion, something not required of adult epidermis. It then seems antithetical that K5 and K14 expression could both be governed by morphology and simultaneously restricted to a single layer of developing cells.

In this report, we address many of the apparent discrepancies that have arisen between studies on epidermal morphology and those on the biochemistry of skin development. To reevaluate these issues, we adapted whole-mount in situ hybridization methods to study mouse embryogenesis from E9 to E16. While this method is not applicable to internal organs, the unique accessibility and architecture of skin provided an opportunity to investigate induction of late stage gene expression in epidermis. We analyzed a series of mRNAs encoding basal keratins, suprabasal keratins and the cornified cell envelope precursor, loricrin. We paralleled these analyses with sensitive assays for a human K5 promoter-driven β-galactosidase (β-gal) transgene. Our studies uncovered a highly specific pattern of keratin expression, which has hitherto gone unrecognized.

We have also investigated transcription factors that might be involved in establishing these patterns of gene expression. In particular, we focused on AP2, a transcription factor of epidermal and neural lineages (Mitchell et al., 1991). Although the precise nature of AP2’s role in epidermal gene expression is not clear, functional AP2 binding sites exist in the 5’ upstream region of many epidermally expressed genes (Leask et al., 1990, 1991; Snape et al., 1990, 1991; Behrens et al., 1991; Byrne and Fuchs, 1993; Rothnagel et al., 1993; Magnaldo et al., 1993; Powell et al., 1992). Here, we report that an AP2 cRNA hybridized in patterns similar to those of basal cell keratin cRNAs. Moreover, we show that expression of AP2 can impart to hepatocytes the ability to express epidermal keratin genes in culture.

MATERIALS AND METHODS

Embryo preparation and β-galactosidase histochemistry

Mice from 3 different heterozygotic lines (Byrne and Fuchs,1993) were mated, and embryo ages were estimated from the time of the appearance of the vaginal plug (=E0.5). Embryos were fixed in 4% paraformaldehyde in PBS for 10-30 minutes depending on age, and washed extensively in PBS. To assay for β-gal activity, whole embryos were incubated in 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) to convert the colorless reagent to a blue end-product. Incubation times for embryos were: 12 hours (E9.5), approx. 2 hours (E10.5-14.5) or 51 hour (E16.5). After post-fixing in 4% paraformaldehyde the embryos were photographed, embedded in paraffin and sectioned (5 μm). Some embryos were embedded in epoxy resin, sectioned (3 μm) and counterstained with toluidine blue. As controls, stainings were sometimes done post-sectioning rather than pre-sectioning.

Preparation of hybridization probes

Digoxigenin eRNA probes were synthesized according to the manufacturer (Boehringer Mannheim Biochemicals, Indianapolis, IN). Biotin cRNA probes were prepared as described by Kopan and Weintraub (1992). A 1900 nucleotide AP2 cRNA probe was made by linearizing plasmid RP1 (Moser et al., 1993; supplied by R. Buettner) with EcoRI and transcribing with SP6 polymerase. An approx. 200 nucleotide AP2B cRNA was made by asymmetric PCR from a PvuI-SpeI AP2B-specific restriction fragment from pNAP2B (Buettner et al., 1993), using the primer AAGAAGGAAGAAGGATGGAG- GTAT. An approx. 1300 nt K14 cRNA was made from HindIII-linearized pl3SP and SP6 RNA pol (Tyner and Fuchs, 1986). An approx. 350 nt K1 cRNA was made from BsrEII-linearized pMK1-3’NC (provided by S. Yuspa). An approx. 220 nt K10 cRNA was made from EcoRI-linearized pMK10-3’NC (provided by S. Yuspa) and SP6 RNA pol. An approx. 800 nt loricrin cRNA was made from BamHI-linearized pGlzskinEE2.9 (H. Hsu and E. Fuchs, unpublished data) and T7 pol.
Whole-mount situ hybridization

In situ hybridizations with digoxigenin probes were as described by Conlan and Rossant (1992) except that proteinase K treatments were optimized to preserve skin morphology without sacrificing penetration of skin by the probes. Treatments ranged from 3.5 minutes in 5 mg/ml proteinase K (E9.5 embryos) to 20 minutes in 20 mg/ml proteinase K (E16.5 embryos). Detection of biotinylated probes was as described (Herrmann, 1991).

RNA analysis

Epidermis was separated from dermis by incubation in 10 mM EDTA in PBS for 10-30 minutes depending on age. RNA was prepared (Chomczynski and Sacchi, 1987), and RT-PCR was performed (Leonard et al., 1993). Primers used were: mouse ribosomal S16 primers (Leonard et al., 1993); AP2A-specific primers, CGG-GTCGCCTGTCGCTCCTCA, TAT-GTCGCCTGTCGCTCCTCA, TTTCTTGCCACTTGCTCATTG; (Lersch et al., 1989), pK14cat2300 (Leask et al., 1990), or using the following per 100 mm dish: 2 pmol of either pK5cat6000 HepG2 (ATCC, Rockville, MD) and SCC-13 (gift of Dr J. Rheinwald) and AP2B, CCCAGCCTCAGCCGCAGCAC, TTACCACGCCAC-TATCCATTTTCCAAGA; primers for the region shared by AP2A equalize DNA levels. 5

al., 1993); and Bluescript-pKS+ (Stratagene, La Jolla, Calif.) to cat and

shown).

expression was consistent with PCR studies, which detected dorsally or ventrally (Fig. 2D,E). The timing of

2C,D). Expression was also induced in limb buds, but was not
detected dorsally or ventrally (Fig. 2D,E). The timing of β-gal expression was consistent with PCR studies, which detected basal keratin mRNAs at low levels as early as E9.5 (not shown).

RESULTS

Basal epidermal transcription is induced early in post-gastrulation mouse embryos: evidence for mesenchymal cues

Previously, we used a promoter from a basal cell epidermal keratin to examine expression of a reporter gene in adult transgenic mice (Byrne and Fuchs, 1993). In three independently derived lines, 6000 bp of human K5 promoter faithfully directed expression of β-gal to basal cells of stratified squamous epithelia, including epidermis and the ORS of hair follicles (Fig. 1). We took advantage of the extreme sensitivity of this system to assay activity of the K5 promoter during mouse embryogenesis. All results outlined below were highly reproducible, not only for the three lines, but in addition, for different litters of the same age within a single line.

K5 promoter activity was not detectable before E9.5. At this time, faint β-gal activity was detected in the dorsolateral region of what appeared to be ectoderm over posterior somites (Fig. 2A). Although difficult to demonstrate by photography, faint expression over the head was also detected. By E10.5, expression was intense in the first branchial arch (Fig. 2B). One day later, β-gal activity included the maxillary and mandibular region of the arch, and also broadened rostrally and caudally in the putative dorsolateral ectoderm over the somites (Fig. 2C,D). Expression was also induced in limb buds, but was not detected dorsally or ventrally (Fig. 2D,E). The timing of β-gal expression was consistent with PCR studies, which detected basal keratin mRNAs at low levels as early as E9.5 (not shown).

To verify that the patterns of K5 promoter activity reflected those of the endogenous K5 gene, we conducted whole-mount in situ hybridization using a digoxigenin-labeled riboprobe specific for K5 (Lersch and Fuchs, 1988). This technique was not sufficiently sensitive to localize the E9.5 K5 mRNAs detected by PCR. However, beginning at E10.5 and thereafter, K5 hybridization was detected, and it increased with age. In contrast, a sense strand K5 control cRNA remained negative throughout embryogenesis (not shown). Throughout E10.5 and subsequent stages of embryogenesis, human K5β-gal and mouse K5 mRNA patterns were indistinguishable. An example of this is shown in Fig. 2H, where K5 cRNA hybridization in E11.5 ectoderm over somites paralleled β-gal staining (compare with Fig. 2F,G). For most data from E10.5-E12.5, we present β-gal staining, since this method was significantly more sensitive.

Sections of stained E9.5 mouse embryos confirmed that β-gal expression was in the ectoderm overlying somites. Morphology was best visualized in semithin sections (Fig. 2I), while K5 promoter activity was best detected in frozen sections (Fig. 2J). The striped pattern of β-gal in this region did not reflect obvious differences in morphology within the ectoderm nor did it appear to be an artifact of the folding of the embryonic surface (Fig. 2K, embryo surface; Fig. 2L, after sectioning; arrowheads provide reference points for comparisons). Rather, expression appeared to be in specific zones, at a time when mitoses were still occurring parallel to the basal lamina (Fig. 2M, bracket; see also Smart, 1970), and when the somitic ectoderm was still single-layered (Fig. 2I).

In contrast to somitic ectoderm, E9.5 head ectoderm was bilayered (Fig. 2N; arrowheads denote basement membrane). Surprisingly, cells from both layers expressed K5β-gal, although initial expression was extremely low and mosaic (bracket). These data revealed for the first time that periderm expresses basal epidermal keratins. While we cannot unequivocally rule out the possibility that the mosaicism was artifactual, we think this is unlikely, since (1) expression in both periderm and ectoderm soon became uniform and persisted throughout development (see below), and (2) mosaicism would be predicted at a time when critical levels of positive acting factors are beginning to accumulate.

A review of expression patterns revealed that K5 was detected at times when mesodermal cells were beginning to populate skin. Expression was seen first in posterior somitic ectoderm, where it then expanded anteriorly and posteriorly. Expression did not seem to be in synchrony with the underlying wave of migration of newly differentiated somitic dermamyotome. Rather, it seemed that it was the nature of the maturing dermamyotome, not migration per se, that provided K5 inductive cues. This was consistent with recombination experiments showing that dermatoic cells from different somites possess distinct properties (Sengel, 1990).

Somites had migrated and differentiated into dermamyotome by the time K5 was detected (Fig. 2I, the 4-5 mesenchymal layers under the ectoderm). Intriguingly, the striped pattern of β-gal expression in the E9.5 ectoderm overlying this region resembled that of underlying homeobox Mox1 gene expression in the E8.5 dermamyotome (Candia et al., 1992). Such mirror image patterns of gene expression in dermamyotome and ectoderm further strengthened the notion that the
striped patterns of K5 promoter activity were genuine and reflective of dermamyotome signals.

K5 induction in the first branchial arch ectoderm also followed soon after derivation and migration of underlying mesenchyme, in this case stemming from neural crest (E10.5; see Fig. 2B; see Nichols, 1981; Couly et al., 1992). In contrast, K5 had not yet been induced in ventral ectoderm, whose underlying mesenchyme derived from somatopleural mesoderm (Sengel, 1976; 1990). Collectively, these data predicted that (1) the K5 promoter in ectoderm receives inductive signal(s) from dermal cells of specific embryonic origin, (2) the inductive cue(s) is acquired in a temporal fashion, and (3) induction does not correlate with morphology, but rather can occur at either the single or bi-layered epithelial stage, and in both ectoderm and periderm, depending on location.

**Patterning of K5 gene expression at E12.5:** correlation with proliferative potential and not stratification

K5 expression in the maxillary region appeared before the precocious development of whisker pads, and by E12.5, it was prominent in upper jaw (Fig. 3A-D). Expression also occurred laterally along the body, but was still reduced ventrally and dorsally (Fig. 3B,C). At E12.5, K5 was not detected in coat, i.e. pelage follicles, whose development is known to lag behind that of tactile vibrissae.

In E12.5 whisker pads, there was no strict correlation between ectodermal morphology and K5 expression. Traces of maxillary and frontonasal fusion were accentuated by a marked absence of K5 in the nasolacrimal groove (arrowhead in Fig. 3D). Both epidermis and the groove side of vibrissae remained negative in a slightly older littermate (Fig. 3E), and yet were morphologically indistinct from surrounding β-gal positive regions.

Vibrissae follicles display a hierarchical developmental sequence, so that a series of embryonic stages can be observed within a single whisker pad (Van Exan and Hardy, 1980 and references within). Vibrissae placodes in decreasing stages of maturation are situated on mesenchymal ridges (Van Exan and Hardy, 1980), and these stained positive for β-gal (left to right in Fig. F). Surprisingly, K5 promoter activity was intense throughout all layers of newly stratified ectoderm (Fig. 3F-I). Numerous suprabasal mitoses were seen, often with a plane of cleavage parallel to the basal lamina (arrowhead in Fig. 3H). In contrast, in single layered ectoderm (see Fig. 2M) and in adult epidermis, the plane of mitotic cleavage is vertical to the basal lamina (Smart, 1970; Weiss and Zelickson, 1975).

Over other body regions, E12.5 ectoderm displayed reproducible, but varied patterns of β-gal activity (Fig. 3J; horizontal section of mid-body). Some areas of forelimb ectoderm displayed equal periderm and ectoderm staining (Fig. 3Ja), while others showed higher periderm than ectodermal staining (Fig. 3Jb). In transitional areas, β-gal was higher in some regions than others (Fig. 3Jc), and absent in others (Fig. 3Id-e). In these regions, there was greater morphological heterogeneity in underlying mesenchyme than in ectoderm.

To summarize our E12.5 data, while K5 induction seemed to correlate with mesenchymal cues, maintenance of K5 expression correlated with proliferative capacity, and a basal-like cell morphology, but not with stratification per se. This parallel could only be recognized through whole-mount studies, and had not been appreciated previously.

**Basal keratin gene expression during pelage follicle initiation: early signs of epidermal differentiation**

By E13.5, K5 promoter activity occurred in most lateral areas (Fig. 4A), but it still lagged dorsally (Fig. 4B) and ventrally (not shown). Intensity of activity continued to be greatest over vibrissae, and the first signs of activity in pelage follicles appeared in the upper dorso-lateral region (arrowheads in Fig. 4B). Body ectoderm was now partially stratified (Fig. 4C-F), but mitoses and β-gal activity still occurred suprabasally, and morphological signs of differentiation were absent.

A dramatic increase in K5 promoter activity took place between E13.5 and E14.5 (compare Fig. 4A with G). Dorsolateral placode condensates stained intensely at early stages of pelage follicle development (Fig. 4H). This gave the embryo surface a spotted appearance, which was readily visible in both β-gal stained embryos (Fig. 4G and inset in 4H) and in whole-mount in situ hybridizations with either K14 or K5 cRNAs (Fig. 4I; shown are K14 cRNA data). Even at this late stage, K5 and K14 cRNAs did not hybridize appreciably to dorsal epidermis over the spinal chord (Fig. 4J), nor to ventral epidermis (not shown).

From E13.5 to E14.5, vibrissae development progressed dramatically (Fig. 4K-N). Throughout immature hair germs, K5 gene expression was uniform (Fig. 4K). As development
proceeded, a region of K5-negative cells accumulated over the invaginating epithelium (Fig. 4L-M). These pockets appeared to be morphologically differentiated, showing no signs of mitoses. Thus in this region, K5 downregulation correlated with a loss of basal-like characteristics and with induction of differentiation.

Follicle cells at the base of the hair germs, i.e. in contact with mesenchymal aggregates, also showed a reduction in β-gal activity (Fig. 4N), a feature seen irrespective of whether skin was sectioned prior to or after the X-gal assay. These results were consistent with previous studies showing that these cells do not crossreact with an anti-K14 antisemum (Kopan and Fuchs, 1989). However, in contrast to K5-negative cells in the upper follicle segment, cells in the lower segment
Fig. 3. Detection of K5 promoter activity in E12.5 embryos. (A-C) Whole-mount staining for K5 promoter activity (A, lateral; B, ventral; C dorsal view). (D) β-gal in whisker pad. Arrowhead points to nasolacrimal groove, negative for β-gal. (E) Lack of β-gal, seen in D, is maintained in slightly older littermate (arrowhead points to site of former nasolacrimal groove). Note presence of vibrissae placodes flanking the groove, which are partly K5 positive and partly K5 negative. (F-I) Sections through whisker pad showing K5 positive, stratified interfollicular epidermis and vibrissae placodes on mesenchymal ridges in decreasing order of maturation. White arrowhead (H) shows mitosis where plane of cleavage is horizontal to basal lamina. (J) Transverse section through upper body of E12.5 embryo. Regions that are magnified in (Ja-Je) are boxed. Note differences in β-gal staining without appreciable variation in ectodermal morphology (Ja-Jc). Note: β-gal was barely detectable in thin epidermis above neural tube (Jd) and ventral epidermis (Je). Bar: 110 µm for F, 35 µm for G and I, 15 µm for H, 555 µm for J and 55 µm for Ja-e.
Fig. 4. K5 promoter activity and basal keratin mRNA expression from E13.5 to E14.5. β-gal activity in lateral (A) and dorsal (B) E13.5 transgenic embryo. Note first indication of pelage follicles, which stain intensely (arrowheads). (C-F) Lateral body skin sections showing stratification and very weak β-gal in inner, middle and outer layers, often most prominent in periderm. Suprabasal mitoses, where plane of cleavage is parallel to basement membrane, are denoted by arrowheads in E. (G) β-gal activity in E14.5 embryo. (H) Section of pelage follicle; inset denotes magnified embryo surface of region sectioned. (I) In situ hybridization with K14 cRNA. (J) Dorsal whole mount showing absence of β-gal over E14.5 neural tube. (K-M) 5 µm paraffin sections through E14.5 vibrissae follicles of increasing maturation. (O-Q) Body skin sections of E14.5 β-gal embryo. Note that in O, K5 promoter activity has diminished in the middle layer of epidermis, where morphological differentiation has occurred. Note that in P, expression is still prominent in epidermal layers where stratification has occurred without morphological differentiation. (Q) In situ hybridization of a K5 cRNA probe against a similar skin section as in P demonstrating the equivalence of the two procedures. Bar represents 35 µm for C, H, K-M; 22 µm for D-F; 53 µm for N and 24 µm for O-Q.
Fig. 5. Localization of keratin and loricrin mRNAs from E13.5 to E16.5. (A-C) E13.5 embryos hybridized with K5, K1 and loricrin cRNAs, respectively. (D-F) E14.5 embryos hybridized with K5, K1 and loricrin cRNAs, respectively. (G,H) Close-up of vibrissae follicles of E14.5 embryos hybridized with K5 and K1 cRNAs, respectively. (I-K) E15.5 embryos hybridized with K5, K10 and loricrin cRNAs, respectively. (L) E16.5 embryo hybridized with loricrin cRNA. (M-P) 10 µm frozen sections from E15.5 embryos. Sectioning was performed after whole-mount in situ hybridization. (M) Localization of K5 mRNA (blue) to basal layer of stratified epithelium and K1 mRNA (brown) to suprabasal layer at E15.5. (N) K5 mRNA in basal layer and in periderm of ventral skin. (O) K10 mRNA in suprabasal layers of backskin. Arrowheads denote basement membrane. (P) Loricrin mRNA over a pelage follicle (backskin). Expression is confined to an upper layer of cells and there is, as yet, no apparent involvement in the follicle. Bar represents 20 µm for M and 35 µm for N-P.
Gene expression in developing epidermis did not appear morphologically differentiated. Thus, even within a single follicle, two different regulatory mechanisms appeared to be operating on K5 gene expression.

At E14.5, interfollicular epidermis began to show signs of suprabasal differentiation, and in these regions, K5 became restricted to the innermost layer (Fig. 4H, O). At this stage, differentiation was regional, and much of the epidermis still showed basal-like cells in the suprabasal layers and concomitant K5 promoter activity (Fig. 4P). These differences were also reflected in analyses of sections from our whole-mount in situ hybridizations (Fig. 4Q).

Expression of K1, K10 and loricrin as epidermal cells begin to differentiate into spinous and granular-like layers

Previously, K1 and K10 expression had been detected as early as E15.5 (Greer and Roop, 1991), with loricrin mRNAs in E16 skin (Yoneda and Steinert, 1993). To assess whether these mRNAs might also display patterning, we subjected whole embryos to in situ hybridizations with K1, K10 and loricrin cRNAs. All three mRNAs were first detected at E13.5 in the nasal region (Fig. 5B, C). At this time, K1, K10 and loricrin cRNAs did not hybridize over dorsolateral epidermis, strengthening our previous conclusion that stratification precedes differentiation in this region. By E14.5, K1 and K10 cRNAs hybridized to whisker pads and paws (Fig. 5E), while loricrin cRNAs hybridized strongly to ventral and lateral body skin but not appreciably to whisker pads (Fig. 5F). These hybridization patterns were specific, and were not observed with sense strand probes.

In vibrissae, K5 and K14 cRNAs hybridized to outer regions (Fig. 5G), while K1 and K10 cRNAs hybridized to inner regions (Fig. 5H). Sectioning (not shown) provided additional biochemical data to support our prior suggestion that K5/K14 negative cells overlying developing placodes were differentiated, and that the switch in keratin expression correlates with differentiation, not stratification.

Consistent with previous PCR and northern analyses (Greer and Roop, 1991; K. Turksen and E. Fuchs, unpublished data), expression of differentiation-specific keratin mRNAs rose dramatically at E15.5 (Fig. 5, compare J with E), and loricrin mRNA rose at E16.5 (Fig. 5, compare L with K; Yoneda and Steinert, 1993). These increases took place over most of the embryo surface, with somewhat lesser hybridization dorsally. By E15.5, ventral skin showed confinement of basal keratin mRNAs to the innermost basal layer and the outermost periderm layer (Fig. 5N) with the differentiation-specific keratin mRNAs confined to suprabasal layers (Fig. 5M, double label, K5 in blue and K1 in brown; Fig. 5O, K10; backskin). Loricrin cRNA was confined to the layer just beneath periderm (Fig. 5P; backskin). This appeared to be the granular layer, known to develop at this time (Sengel, 1976).

We were surprised that loricrin cRNA hybridized over pelage follicles, since sections showed no hybridization within follicles. The patterning may be reflective of pre-inductive events for inner root sheath (IRS) formation: we have detected loricrin mRNA in adult follicle IRS (data not shown), despite negative data to this point from other groups (Hohl et al., 1991; Yoneda and Steinert, 1993).

E16.5: the last day at which whole-mount in situ hybridization can be used to track epidermal markers

By E16.5, last vestiges of K5-negative dorsal skin disappeared, and K5 promoter activity occurred over the entire embryo surface (Fig. 6A, B). Interfollicular epidermis maintained basal-specific expression, while pelage follicles displayed K5 patterns reflecting a range of stages similar to that seen earlier in whiskers (Fig. 6C-F). By this time, K5 expression was prominent in the outer follicle layer (Fig. 6E), probably predicting the eventual confinement of K5 follicle expression to the ORS (Fig. 1). K5 gene expression was down regulated in the vicinity of dermal papillae (Fig. 6F), in a fashion analogous to that seen in vibrissae (Fig. 4M, N). After E16.5, as a stratum corneum developed, skin became impermeable to these probes.

AP2 cRNA hybridization during development

K5 and K14 promoters share a series of transcription factors, including AP2, Sp1 and protein 1/2 (Byrne and Fuchs, 1993).
AP2 was of particular interest, not only because of functional AP2 binding sites in a variety of epidermal promoters, but also because of its enriched expression in epidermis (Leask et al., 1990; Snape et al., 1990; Mitchell et al., 1991). In contrast, Sp1 had limited value in relating AP2 cRNA hybridization to K5 and K14 patterns.

As shown in Fig. 8, AP2 cRNA patterns were remarkably similar to those of K5 and K14 cRNAs. At early stages, AP2 cRNA localization reflected somite organization (Fig. 8A, AP2, compare with 8B, K5), as well as prominence in the first branchial pouch (Fig. 8C, compare with 2B, K5). By E12.5, hybridization extended over the dorso-lateral surface, and was diminished in intensity during development, as keratin expression was rising (Fig. 7B). A lower band appeared prominent at later stages of development, when K5 and K14 gene expression was rising (Fig. 7B). The lower band was detected in E13.5-E16.5 skin when extended cycling times were used. Overall, while there may be additional species of AP2-like mRNAs in mouse skin during development (see also Winning et al., 1991; Buettner et al., 1993), of the two known AP2 variants, only AP2A mRNAs seemed to correlate with K5 and K14 gene expression.

One way to explore further whether AP2 or AP2-like factors might be playing a significant role in keratin gene expression is to assess whether mRNAs hybridizing to an AP2 cRNA display pattern formation similar to that of keratin cRNAs. To investigate this possibility, we subjected our embryos to whole-mount in situ hybridization with a mouse AP2 cRNA that recognizes both AP2A and AP2B forms. Previously, Mitchell et al. (1991) had conducted in situ hybridizations with this probe on E8.5-E12.5 embryo sections, and noted hybridization in surface ectoderm at E8.5, i.e. one day before we detected K5 ß-gal activity in these cells. Beyond this observation, conventional methods were of limited value in relating AP2 cRNA hybridization to K5 and K14 patterns.

Sectioning of hybridized embryos revealed that AP2 RNAs were largely ectodermal over much of the body surface. This included intense ectodermal hybridization in limb skin (Fig. 8I). Interestingly, the spots of AP2 mRNA that were seen over the dorso-lateral surface, and was detected in vibrissae (Fig. 8D). At E13.5 and E14.5, parallels between K5 and AP2 cRNA hybridizations were particularly striking (Figs. 8E-H). A major difference was in fore and hindlimbs, where K5 hybridization was not as prominent as AP2 (Fig. 8E, compare with 8F,G). Intriguingly, AP2 cRNA hybridization to pelage follicles at E13.5 (Fig. 8G) preceded hybridization of K5 and K14 cRNAs to these areas (compare with Fig. 4A).

Further sectioning revealed an even more complex pattern of AP2 hybridization at E13.5. In some regions, e.g. whisker pad, AP2 patterns were remarkably similar to K5 (8K-M).
other regions, e.g. upper and lower jaw, paws and tail. AP2 cRNAs hybridized to both mesenchymal and ectodermal cells, a feature also noted by Mitchell et al. (1991) (Fig. 8N). This mesenchymal AP2 hybridization seemed to be due to AP2B, as judged by hybridization with a specific probe (Fig. 8O and P). Often, however, the total probe showed a hybridization
pattern similar to K5, which likely reflected the pattern of AP2A-like factors (Fig. 8Q).

By E15.5, as the skin became both stratified and differentiated, hybridization detected by the general AP2 probe appeared regionally confined to basal cells (Fig. 8R and S), similar to K5. Collectively, our data suggested that AP2, but not AP2B, preceded slightly and was strikingly similar to the pattern of K5 gene expression in the developing embryo.

AP2 can impart to hepatocytes the ability to express K14 and K5 genes in culture

The correlation between AP2A and basal keratin cRNA hybridizations prompted us to explore whether AP2 or an AP2-like factor might be sufficient to impart to other cell types the ability to express K5 and K14. We focused on the human liver hepatocyte line, HepG2, which normally expresses K8 and K18, but not K5, K14, or AP2 (Williams et al., 1988). As test genes we used pK5cat6000 and pK14cat2300, containing 6000 and 2300 bp, respectively, of human K5 and human K14 5′ upstream sequence (Leask and Fuchs, 1990; Byrne and Fuchs, 1993).

On their own, K5 and K14 promoters generated minimal cat expression in HepG2 cells (Fig. 9A and B, respectively, set 1). In contrast, these promoters produced marked expression in human epidermal (SCC13) keratinocytes, which express endogenous K5 and K14 and which have abundant AP2 activity (Leask et al., 1990). When a human AP2A expression vector (Buettner et al., 1993) was co-transfected with pK5cat6000, a substantial increase (up to 18X) in cat expression was observed in HepG2, but not in SCC13 (Fig. 9A, sets 2-4). Similarly, up to a 44× increase in cat expression was observed when HepG2 cells were co-transfected with pK14cat2300 and the AP2A vector (Fig. 9B, sets 2-4). At high levels of AP2A, the response was reduced, suggestive of squelching. The concentration needed to see squelching varied with promoter and cell type, and the effects could not be explained by variation in tk-β-gal control expression.

When pK5cat6000 was co-transfected with an expression vector encoding the dominant negative AP2B, K5 promoter activity remained low in HepG2 cells and was suppressed in SCC13 cells (Fig. 9A, set 5). For the K14 promoter, AP2B had no apparent effect in HepG2 cells, and in SCC13 cells, it appeared to relieve the squelching described above (Fig. 9B, set 5).

When these experiments were repeated using p5xTREcatTK-CAT, containing 5 repeats of an AP1 site linked to the Herpes thymidine kinase promoter (Angel et al., 1988), no appreciable AP2A-mediated enhancement in HepG2 cells was observed (not shown). Collectively, our results demonstrate that a simple epithelial cell, which is non-permissive for epidermal keratin gene expression, can be converted to a permissive cell by expression of AP2A. These data are also consistent with our PCR results, and suggest that as ectodermal development proceeds and K5 and K14 expression rise, positive acting AP2 forms are likely to prevail in basal cells. Testing this prediction will require the cloning of all AP2-like forms and the development of monospecific AP2 antibodies.

DISCUSSION

Our investigation of human K5-β-gal transgene activity indicate that (1) sequences involved in the complex developmental program of basal keratin expression are contained within 6000 bp of 5′ upstream region of the human K5 gene, (2) these sequences are evolutionarily conserved and (3) β-gal pattern formations are bona fide representations of the patterns of K5 and K14 gene expression. The high sensitivity of the β-gal assay made it ideal for early embryonic studies, when mRNA and protein expression levels are extremely low. While we cannot rule out minor differences between β-gal and endogenous K5 gene expression, we did not detect such differences at times (E10.5-E16.5) where data with both probes
could be analyzed. When coupled with whole-mount in situ hybridization, the approach enabled us to uncover novel and hitherto unexplored patterns of gene expression in embryonic development. The existence of epidermal patterning underscores the importance of whole-mount analyses in evaluating programs of gene expression in skin, and exposes the limitations of conventional in situ hybridizations, and of mRNA or protein analyses of whole embryo (or organ) extracts.

Early patterns in K5 gene expression seem to be established from inductive cues imparted by underlying mesenchyme and can occur in a single-layered ectoderm

Many studies have focused on the role of dermis in the formation of epidermal appendages such as hair follicles, feathers and scales (for reviews, see Sengel, 1986; 1990; Hardy, 1992). The specification of these structures occurs relatively late in embryogenesis and depends upon signals imparted by dermis of diverse embryonic origins (Dhouverly et al., 1978; Van Exan and Hardy, 1984). The precise nature of the dermal-epidermal signals has not yet been determined, though a number of proteins demonstrate regional microheterogeneities in the vicinity of developing appendages (Sengel, 1990; Holbrook et al., 1993: present studies). Typically, such differences have been noted for dermal development post E13, when the round, immature mesenchymal cells have differentiated (Van Exan and Hardy, 1984).

A long-standing question has been whether the capacity to synthesize epidermal keratins is conferred by underlying mesenchyme or whether it is acquired by self-determination (for review, see Sengel, 1986). Our studies demonstrate that the patterning and timing of K5 induction coincide with well-known regional heterogeneities in the origins of dermal mesenchyme, suggesting that the major K5 inductive cues come from developing dermal cells. Cues appear as early as E9.5, i.e. significantly earlier in epithelial morphogenesis than had been suspected. In fact, in some regions, notably the ectoderm overlying somites, K5 gene induction even precedes formation of a columnar basal layer. This sets K5 induction as a very early biochemical marker of epidermal development and among the earliest consequences of dermal-epidermal interactions thus far described. These findings necessitate revision of prior assumptions that epidermal keratin induction correlates with the morphogenesis of a bilayered epithelium (Jackson, et al., 1981; Moll et al., 1982; Dale et al., 1985; Kopan and Fuchs, 1989).

What might be the biochemical natures of the inductive dermal signals? At the moment, it is too early to say. However, it is interesting that a number of Hox genes show patterns of expression that overlap partially with and are either parallel to, or mirror image to, those of the epidermal keratin genes (Hunt et al., 1991; Candia et al., 1992; Takahashi et al., 1992). Further studies will be necessary to explore these issues.

Periderm formation and K5 expression

The periderm has been a developmental enigma for many years. Most researchers agree that periderm stems from upward migration of ectoderm. This said, histological studies reveal mitoses in this layer (Herken and Schultz-Ehrenburg, 1983), and morphological distinctions between periderm and underlying embryonic basal layer have led to suggestions that amniotic fluid rather than ectoderm and/or dermis may orchestrate the program of gene expression in these cells (for discussion, see M’Boneko and Merker, 1988). Our studies add three findings that underscore similarities between periderm and immature ectoderm: (1) K5 gene expression in single layered ectoderm, (2) K5 gene expression in periderm cells, and (3) coexpression of K5 in periderm and in embryonic basal cells in a highly patterned fashion. Hence, periderm not only appears to arise from stratification of a single layered ectoderm, but also it receives inductive cues for gene expression from the same source as ectoderm.

Expression of K5 and K14 correlates with proliferative activity of developing basal cells, not with stratification

K5 and K14 expression clearly correlate with proliferative activity of cells in embryonic skin and not stratification, and K5 and K14 mRNA downregulation correlate with differentiation and not stratification. While these findings are in contrast to earlier developmental studies on keratins, they are consistent with morphological studies noting major differences between embryonic and adult stratification (Smart, 1970). Discrepancies from past keratin studies can be explained by (1) the failure to appreciate that keratin gene expression is highly patterned in embryonic skin, in a fashion which at early stages does not correlate with cell morphology, and (2) the frequent use of monoclonal antibodies, which were often-times not only cross-reacting, but also prone to antigen masking, a feature that is now a well-known problem in anti-keratin immunohistochemistry.

In our studies, we compared K5 and K14 mRNA expression from E13 to E16.5, and throughout this time, patterns were indistinguishable. In human embryogenesis, K5 expression has been reported to precede that of K14 (Moll et al., 1982). Due to the relatively short gestation period of mouse compared to human, it was not possible to assess whether such a difference also occurs during mouse embryogenesis.

Expression of differentiation-specific keratins and loricrin

Many studies correlate K1 and K10 induction with formation of a three-layered epithelium in mammalian skin (Moll et al., 1982; Dale et al., 1985; Kopan and Fuchs, 1989). However, it is clear from whole-mount studies that K1 and K10 mRNA expression correlates with differentiation, not stratification. This process occurs well after somitic and neural crest differentiation to mesenchyme, and hence, early regulatory cues seem to be quite different for the two keratin gene pairs. In contrast, some later cues may be shared, as judged by regional similarities in expression patterns post E13.5.

The timing of differentiation-specific markers was markedly earlier than previously suspected. This was largely because K1 and K10 appear much earlier in some regions e.g. nasal epithelium, than in backskin, i.e. the skin source for most prior investigations. While K1 and K10 showed regional inductive patterns that were similar to those of K5 and K14, loricrin diverged considerably, suggesting cues for its expression may differ at least in part from those of the keratins.

AP2 and basal epidermal gene expression

Our compilation of expression patterns of epidermal mRNAs
during embryonic development should be useful for dissecting out the complex mechanisms underlying epidermal development and differentiation in mammals. We have begun to test this notion by exploring the possible physiological relevance of AP2 binding sites in the 5′ upstream regulatory regions of many epidermal genes.

Multiple forms of AP2 appear during skin embryogenesis (Winning et al., 1991; Snape et al., 1991; Buettner et al., 1993), and in mouse, a cRNA corresponding to a large coding segment of AP2 hybridizes to tissues of ectodermal and neural crest lineages, peaking at E11.5 (Mitchell et al., 1991). Our study extends AP2 analyses up to E15.5, and further suggests that AP2 cRNA hybridization patterns closely mimic, and precede slightly, those of K5 and K14 cRNAs, while AP2B mRNAs are primarily localized in underlying mesenchyme. AP2-like mRNAs appear to be in the right place and at the right time to play a significant role in controlling basal epidermal gene expression.

Our culture studies provide the first direct demonstration that AP2 can act in a positive fashion to control K5 and K14 gene expression, while AP2B counteracts this positive effect. Moreover, our data show that K5 and K14 gene expression can be induced in cultured hepatocytes when they are co-transfected with an AP2 expression vector. These data don’t necessarily imply that the absence of AP2 in non-K5/K14 expressing cell types is what accounts universally for the absence of keratin gene expression. In fact, we know that HeLa cells express AP2 and yet they still do not express K5-β-gal (Lersch and Fuchs, unpublished data). Additionally, we know that keratinocytes possess other factors required for K5 and K14 expression (Leask et al., 1990; 1991). This said, our studies do suggest that (1) the AP2 sequences that hybridized to embryonic ectoderm are likely reflective of an overall balance that is positive rather than negative for AP2 activity, and (2) AP2 is likely to play a significant role in K5 and K14 regulation in vivo, both with regards to its presence in basal-like epithelial cells and with regards to its absence in some other cell types.

Our PCR analyses indicate that AP2 mRNA patterns may be more complex than previously realized. Further studies will be necessary before we understand fully how this family of mRNAs controls basal keratin gene expression during development. In the future, as expression of other epidermal mRNAs are reevaluated using the approaches described here, we anticipate that new correlations will become apparent and new insights into mechanisms involving epidermal development will emerge.

A very special thank you goes to Dr Qian-Chun Yu who prepared semithin sections, Linda Degenstein for transgenic mouse technology and Jennifer Jacobs who assisted with photography and preparation of embryos. We also thank Dr Brian Aneskievich for discussion and Grazina Traska for help with tissue culture, Phillip Galiga for preparation of art work and Paul Gardner for his help in synthesizing oligonucleotide primers. We thank Dr Pamela Mitchell (Institute for Molecular Biology, University of Zurich, Switzerland) and Dr Reinhard Buettner (Institute for Pathology, University of Regensburg, Germany) for mouse AP2 cDNA clones used in whole-mount in situ hybridizations, Dr Stuart Yuspa (NIH) for mouse K1 and K10 cDNAs. C. B. is a research associate supported by the Howard Hughes Medical Institute. E. F. is an investigator of the Howard Hughes Medical Institute. This work was supported by a grant AR31737 from the National Institutes of Health. The University transgenic facility is in part supported by grant CA 14599 from the National Cancer Institute.

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