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Patterns of epithelial expression of Fos protein suggest important role in the transition from viable to cornified cell during keratinization

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Summary

An antibody directed against the DNA-binding region of c-*fos* was used to localize the distribution of cells positive for Fos protein in epithelial tissues. The antibody consistently bound to the nuclei of epithelial cells in the late stages of differentiation, just prior to cornification. The epidermis, palate, buccal mucosa, gingiva, tongue, forestomach and vagina in estrus all produced this type of labelling, suggesting a burst of expression immediately before cell death and cornification. The differentiating cells of the hair follicle, including the hair and inner root sheath, were also labelled. Non-keratinized tissues including junctional epithelium, embryonic epidermis and diestrus vaginal epithelium showed little or no Fos labelling. With the onset of keratinization at 18 days gestation or with induction of estrus in ovariectomized mice with estradiol benzoate, the epidermis and vagina expressed Fos protein in the manner typical for keratinized tissues. The *Er/Er* mutant epidermis, a tissue that is blocked in its ability to keratinize, overexpresses Fos with Fos-positive cells appearing in virtually every cell layer. Gel shift analysis demonstrates the presence of a functional AP-1 complex in epidermal extracts that is recognized by our antibody. Our data suggest that the expression of Fos is intricately related to epithelial cell differentiation, specifically in relation to the process of cornification and cell death.

Key words: c-*fos*, protooncogene, keratinization, cell death, differentiation.

Introduction

Keratinization is an orderly process involving cellular stratification and differential regulation of epithelial-specific gene products. Keratinizing cells arise from an epithelial basal cell layer and migrate toward the tissue surface. During the course of this migration, the cells undergo a variety of morphological changes that reflect the differential expression of epithelial genes (Fuchs and Green, 1980; Sun *et al.* 1984; Fisher *et al.* 1987*a*,*b*). The final event in this process of cellular differentiation is the cornification of the cell resulting in the elaboration of complexes of proteins and the degradation of cellular organelles (Brody, 1959; Lavker and Matoltsy, 1970; reviewed in Holbrook, 1989). The protein complexes consist primarily of cytoskeletal elements (keratin intermediate filaments) embedded in an electron-dense matrix of protein (Brody, 1959, 1960; Dale *et al.* 1978), and an insoluble, membrane-associated envelope (Rice and Green, 1979). The assembly of these materials is accomplished by the enzymatic modification of proteins present in the viable cells of the epithelium (Resing *et al.* 1984; Bowden *et al.* 1984; Rice and Green, 1979).

The transition from a viable cell, synthesizing epithelial structural proteins, to a dead, cornified cell is dramatic. This process occurs rapidly and likely requires the sudden induction of a host of genes, including nucleases and proteases, in response to an as yet unknown stimulus. The Fos family of proteins are transcriptional activators that are rapidly induced by extracellular stimuli (Turner and Tjian, 1989; Zerial *et al.* 1989; Cohen and Curran, 1988). We are interested in localizing the expression of protooncogenes in epithelia in order to identify the cell types and periods during development that are critical to the appearance of these genes. This paper reports that Fos protein is expressed immediately prior to cell death and cornification in keratinizing tissues. The data suggest that Fos plays an important role in the transcriptional activation of genes mediating cornification and, also, that the cells expressing Fos in keratinizing epithelia are the target of an as yet unknown differentiation signal.

Materials and methods

Animals and tissues

Embryonic and newborn Swiss-Webster mouse and Sprague-

Dawley rat tissues were collected and fixed in 4 % paraformaldehyde in phosphate-buffered saline (PBS). Tissues were embedded in paraffin by routine procedures. In the case of adult vaginal tissue, mice were ovariectomized, allowed to recover for 2 weeks, and then administered 10 μg estradiol benzoate in corn oil *i.p.* This allowed us to collect nonkeratinized (ovariectomized) and keratinized (72 h post-estradiol) vaginal tissue (Barker and Walker, 1966). For examination of adult oral tissues, 4-week-old Sprague-Dawley rats were anesthetized, perfused for 10 min with 4 % paraformaldehyde in 0.1 M phosphate buffer, and jaws removed and fixed for another 1–2 h. Jaws were decalcified for 3–5 days in 4 N formic acid in 0.5 M sodium formate at 4 °C.

Fos antibodies

A number of antibodies directed against c-*fos* peptides were used in these studies. Two antibodies directed against NH_2-terminal peptides gave high backgrounds in immunohistochemical preparations. These peptides were subsequently found to share significant homology (approximately 40–60 %) with basic keratins and to cross-react with keratins on Western blots. The antibodies that gave the best results were prepared against a 25 amino acid synthetic peptide from the DNA binding region (the M-peptide region; Franza *et al.* 1987) of c-*fos* as previously described (Quinn *et al.* 1989). This peptide is 100 % conserved in the mouse, chicken and human c-*fos* protein (Van Straaten *et al.* 1983; Van Beveren *et al.* 1983; Molders *et al.* 1987). Briefly, the peptide (KVEQLSPEEEKRRIRRERNKMAAA) was conjugated with 1-ethyl-3(3-dimethylaminopropyl)-carbodiimide to succinic anhydride-reacted keyhole limpet hemocyanin and injected intradermally in rabbits in an emulsion containing Freund's complete adjuvant. The antibodies were purified by affinity chromatography with the peptide.

Immunohistochemistry

Immunohistochemistry by the avidin–biotin–peroxidase technique was performed as previously described (Fisher *et al.* 1987*a*) except that the primary antibody (1:1000 dilution) step was carried out at 37 °C for 2–3 h and sections were stained briefly with hematoxylin. Adult rat jaws were equilibrated in 30 % sucrose, serially sectioned at 50 μm with a freezing microtome, and the sections incubated in a 1:2500 dilution of the M peptide antibody for 60 h at 4 °C. Antibody binding was localized by the avidin–biotin–peroxidase technique and sections were counterstained with cresyl violet. Controls, consisting of elimination of primary antibody and competition of antibody with 10^{-6} M peptide, were routinely negative.

Epidermis extracts

Epidermises of newborn (1- to 2-day-old) mice were extracted by a procedure modified from Dignam *et al.* (1983). The buffers were modified as in Quinn *et al.* (1989). In summary, the epidermis was separated from mouse skin after incubation in 10 mM EDTA at 55 °C for 2 min. After separation, all subsequent steps were at 4 °C. The epidermis was minced in the extraction buffer containing 20 mM Hepes (pH 7.2), 20 % glycerol, 0.42 M sodium chloride, 1.5 mM magnesium chloride, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol and 2.1 μg ml^{-1} aprotinin. The tissue was homogenized with 10 strokes, twice, in a Dounce homogenizer with the B pestle and centrifuged at 25 000 *g* for 20 min. The supernatant was dialyzed for 3–4 h against dialysis buffer containing 80 mM potassium chloride instead of the sodium chloride and magnesium chloride in the extraction buffer. The supernatant was rapidly frozen in ethanol/dry ice and stored at −70 °C. These extracts were used in gel retardation studies.

Gel retardation analysis

Gel retardation analysis was performed as previously described (Singh *et al.* 1986; Quinn *et al.* 1989). Briefly, ^{32}P-labelled 3′-end double stranded oligonucleotide representing the gibbon ape leukemia virus (GALV) enhancer (CGAGAATAGATGAGTCAACAGCG) was reacted with varying concentrations of epidermal extracts, with and without anti-M peptide antibody, and run on polyacrylamide gels. Controls consisted of competition with cold GALV oligonucleotide and competition with a random-mer oligonucleotide.

Results

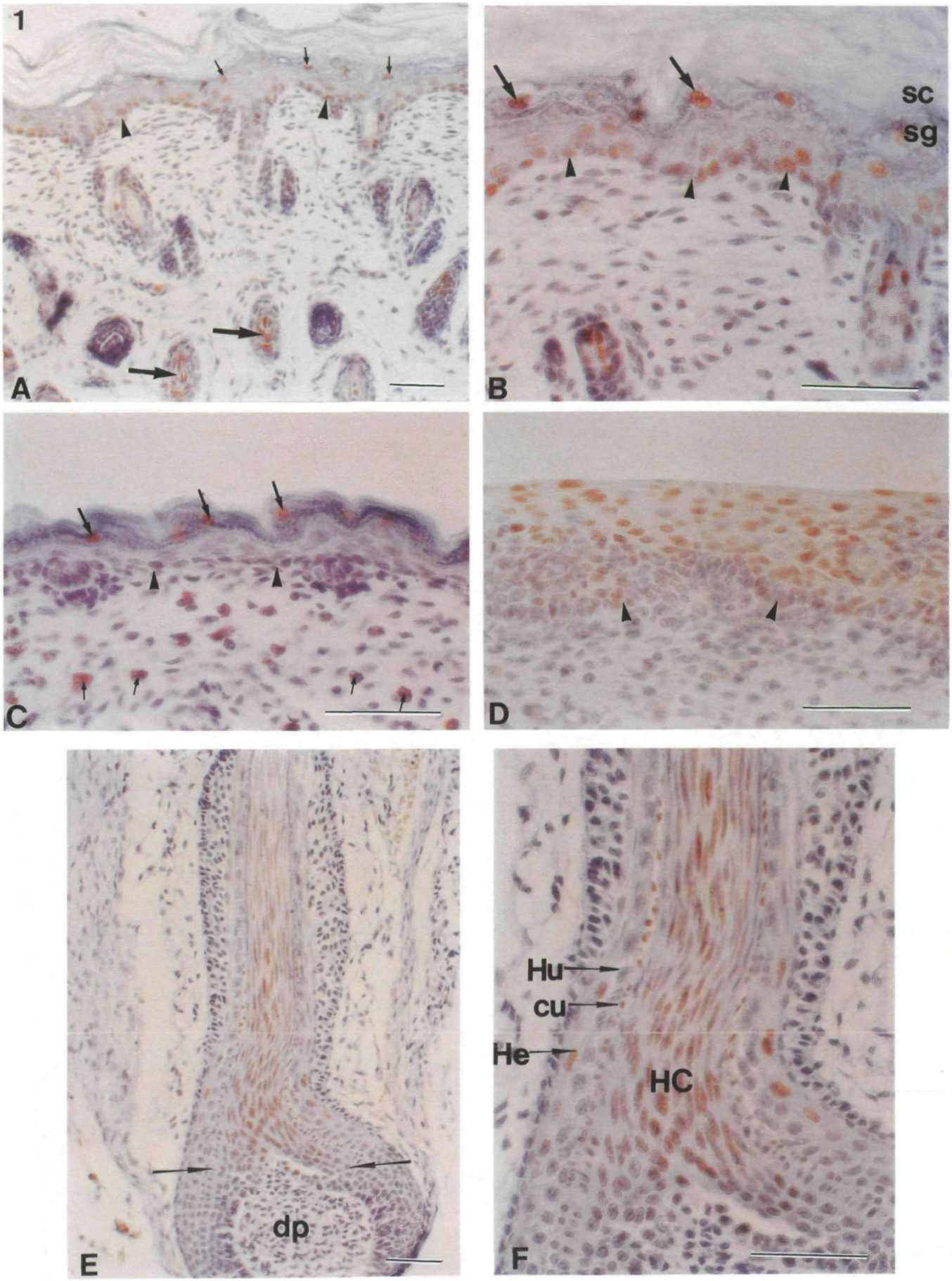
Immunohistochemistry

A number of rodent epithelia were examined for the localization of Fos including skin and hair, oral epithelia, forestomach and vagina from estradiol-induced, ovariectomized mice. Results were identical in every case in which both rat and mouse tissues were examined.

Skin and hair

The localization of Fos-positive nuclei in skin was restricted to the epidermis and the epithelial component of the hair follicle (Fig. 1A,B). Within the newborn epidermis of mice and rats Fos-positive cells were found in the basal layers and the upper granular layers of the epidermis (Fig. 1B). The cells in the upper granular layers showed a more intense immunoreactivity over the immunoreactive cells in the deeper epidermal layers. While the nuclear labelling of the basal cell layer was of variable intensity depending upon the preparation, the labelling of the upper granular layer was consistently and strongly positive.

Fig. 1. Localization of Fos protein in rodent skin.
(A) Neonatal rat skin shows labelling in epithelial cells including basal cell layer (arrowheads), granular cell layer (small arrows) and hair follicles (large arrows). Bar=50 μm. (B) Higher magnification of interfollicular epidermis demonstrating typical Fos labelling in epidermis. Note labelling of basal cell layer (arrowheads) and intensely positive cells (arrows) in the upper stratum granulosum (sg), just below the cornified cells of the stratum corneum (sc). Bar=50 μm. (C) The upper granular layer Fos-positive cells (large arrows) first appear in interfollicular epidermis of 17–18 day gestation embryonic mice. The Fos-negative epidermal basal cells are indicated by arrowheads. Non-specifically stained cells of the dermis, probably mast cells, are indicated by small arrows. Bar=50 μm. (D) The hyperplastic epidermis of the 18 day gestation *Er/Er* mouse shows Fos-positive cells throughout its thickness, in most cell layers. Basal cell layer indicated by arrowheads. Bar=50 μm. (E) The newborn mouse vibrissae follicle has Fos-positive cells in almost all layers of the hair cortex and inner root sheath, beginning (arrows) at the level of the apex of the dermal papilla (dp). Bar=50 μm. (F) Higher magnification of E showing that positive labelling of cells for Fos begins at about the level of the apex of the dermal papilla in all layers of the hair cortex (HC), and in the inner root sheath cuticle (cu) and Henle (He) layers. Fos is not detected in the inner root sheath layer of Huxley (Hu). Bar=50 μm.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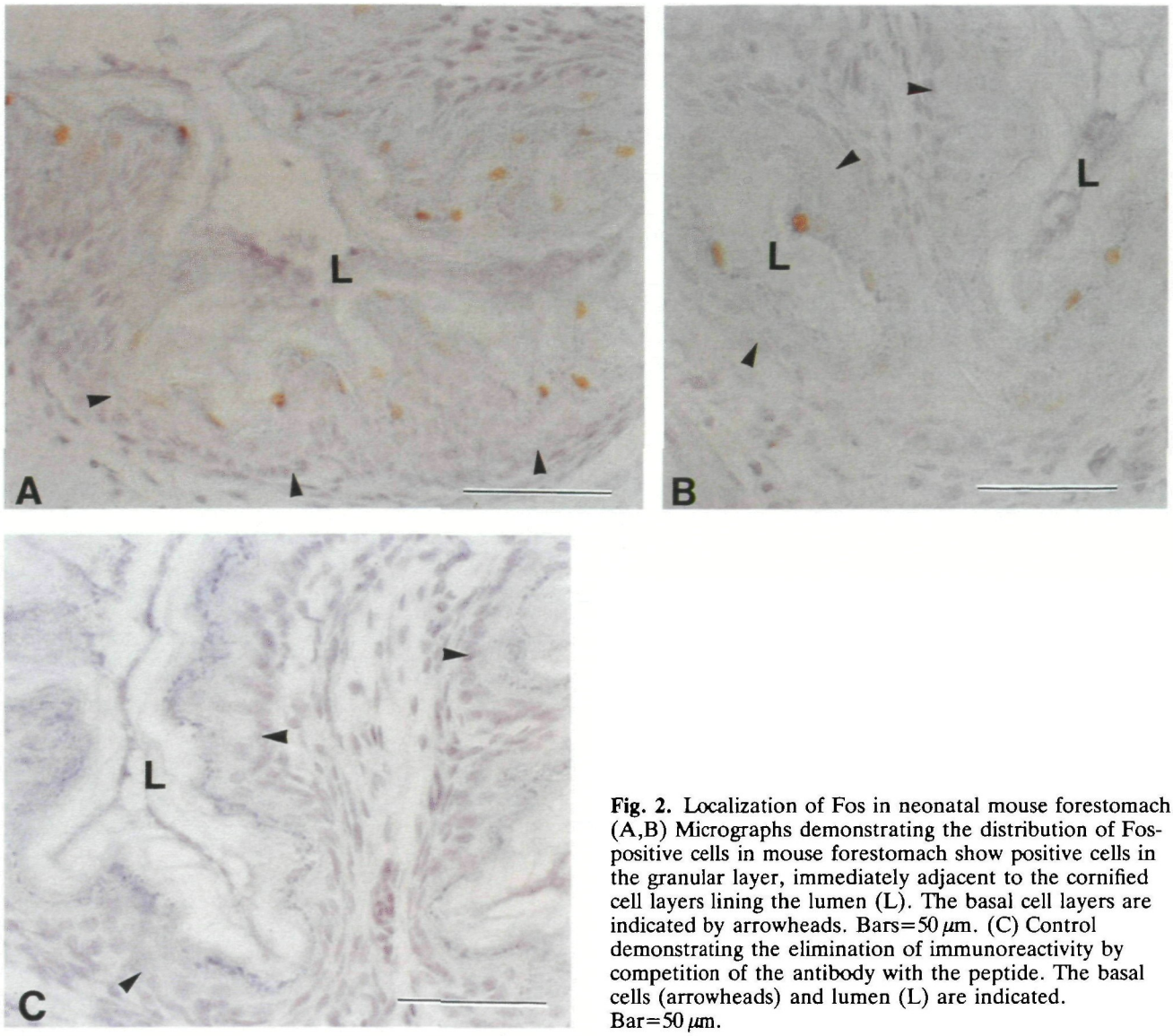

Fig. 2. Localization of Fos in neonatal mouse forestomach. (A,B) Micrographs demonstrating the distribution of Fos-positive cells in mouse forestomach show positive cells in the granular layer, immediately adjacent to the cornified cell layers lining the lumen (L). The basal cell layers are indicated by arrowheads. Bars=50 μm. (C) Control demonstrating the elimination of immunoreactivity by competition of the antibody with the peptide. The basal cells (arrowheads) and lumen (L) are indicated. Bar=50 μm.

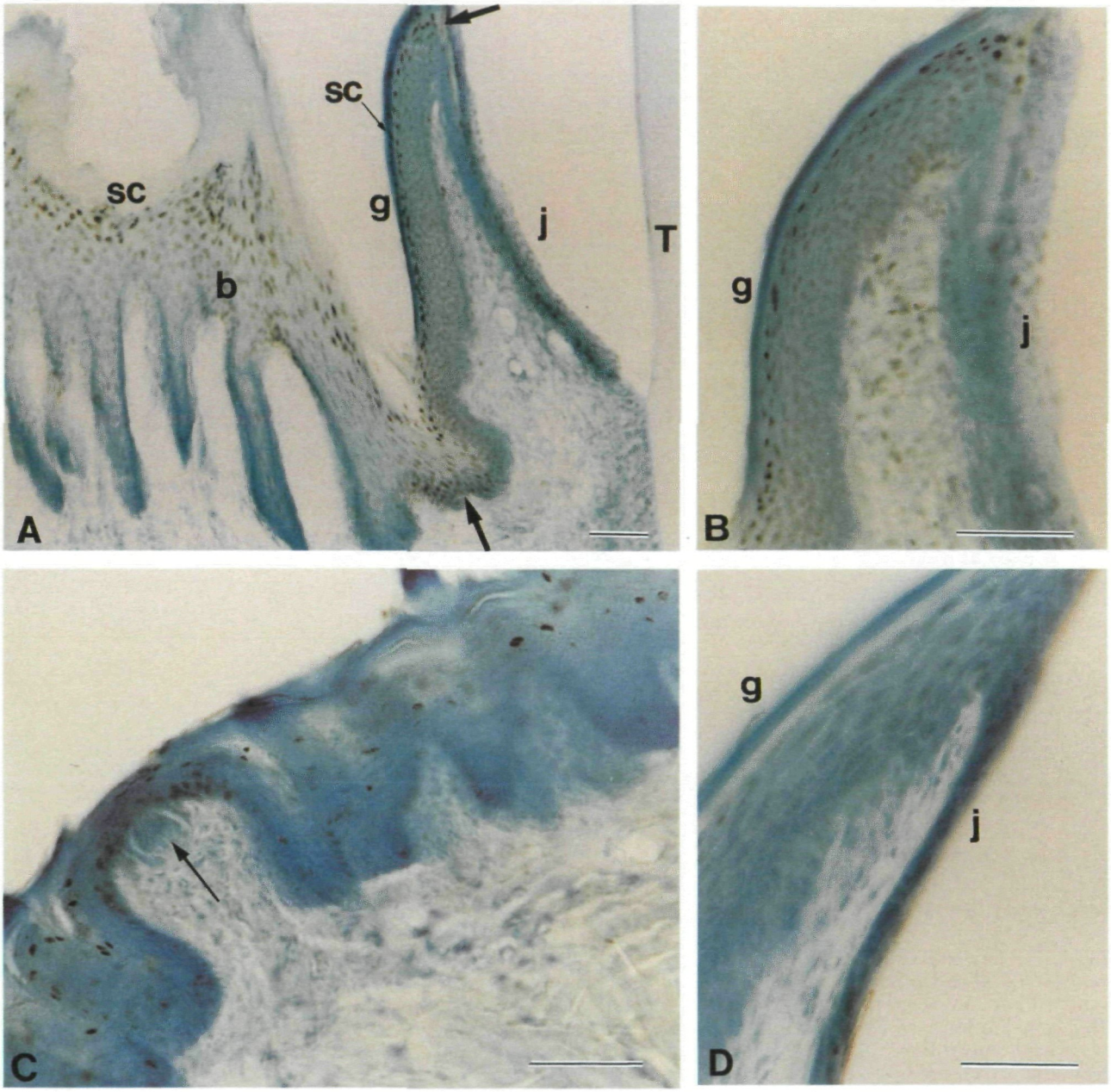

Fig. 3. The localization of Fos-positive cells in rat oral epithelia. (A) Section through oral epithelia of 4-week-old rat demonstrating the localization of Fos-positive cells in buccal (b), gingival (g) and junctional (j) epithelia adjacent to tooth enamel (T). The keratinizing buccal (cut tangentially) and gingival epithelia show numerous Fos-positive cells just below the stratum corneum (sc) while the nonkeratinized junctional epithelium (j) is largely negative. Note the switch in Fos labelling in transition zones between epithelia (arrows). Bar=100 μm. (B) Higher magnification of gingival (g)–junctional (j) epithelial transition showing the lack of Fos labelling in the nonkeratinized junctional epithelia. Bar=100 μm. (C) Micrograph of tongue epithelium demonstrating Fos-positive cells associated with keratinization in outer epithelium. Some basal cell staining is associated with the fungiform papilla housing a taste bud (arrow), but the strongest labelling is associated with keratinization in the filiform papillae flanking the fungiform papilla. Bar=100 μm. (D) Incubation of the antibody with the peptide eliminates labelling in the gingival (g) and junctional (j) epithelia. Bar=60 μm.

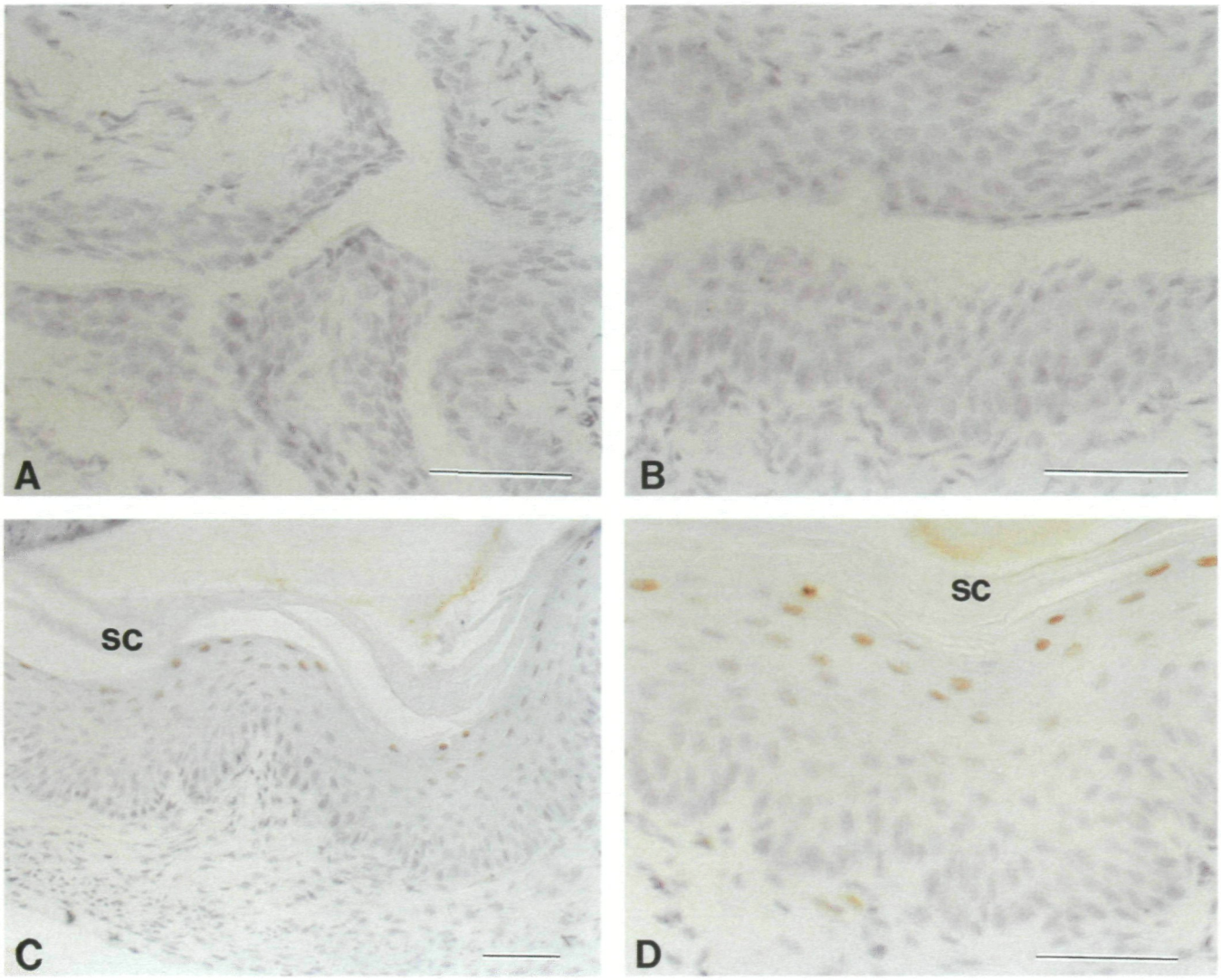

Fig. 4. Fos localization in vaginal epithelium of ovariectomized, estradiol benzoate treated mice. (A) Ovariectomized mice receiving no estradiol show no signs of Fos labelling in the mucous-secreting epithelium. Bar=50 μm. (B) 24 h after receiving estradiol benzoate the mucous-secreting epithelium is still negative for Fos. Bar=50 μm. (C) 72 h after receiving 10 μg estradiol benzoate the vaginal epithelium is now keratinized and shows Fos-positive cells in the outermost layers of the granular layer. Bar=50 μm. (D) Higher magnification of C showing the keratinized vaginal epithelium of an ovariectomized mouse, 72 h after estradiol benzoate injection. Bar=50 μm.

Embryonic epidermis ≤16 days gestation exhibited no or weak immunoreactivity (data not shown). The intense labelling associated with keratinocytes in the upper granular layers was not evident in embryonic epidermis until the onset of keratinization at 17–18 days gestation in the mouse (Fig. 1C). In order to test whether this pattern of nuclear labelling was altered in an epidermis that is blocked in its ability to differentiate, we examined the distribution of Fos in the epidermis of the repeated epilation (*Er/Er*) mutant mouse (Fig. 1D). The *Er/Er* mutant epidermis presented an altered pattern of labelling with virtually all cell layers showing nuclear labelling throughout the thickened mutant epidermis.

Cells immunoreactive with the M-peptide antibody were also detected in both pelage (Fig. 1A,B) and vibrissa (Fig. 1E,F) hair follicles, both in the infundibulum of the hair as well as the hair bulb (Fig. 1A). Preparations of individually embedded vibrissa follicles were useful for accurately localizing the Fos-positive cells in the hair and inner root sheath (Fig. 1E,F). The cells of the hair first expressed Fos as they moved from the proliferative compartment into the zone immediately superior to the dermal papilla. The nuclei remained positive throughout the period of differentiation until the cells cornified higher in the hair follicle (Fig. 1E); by the time the hair keratinized the nuclei became elongated and parallel to the axis of the cornifying hair cell. The cells of the cuticle showed a similar pattern of Fos expression during their differentiation. The small cuticle cell nuclei appeared as a string of small nuclei that were Fos-positive above the level of the dermal papilla and ended with cornification (Fig. 1E,F). Layers of the inner root sheath, on the other hand, showed a more variable pattern of staining with positive cells appearing in the suprabulbar region in the layer of Henle but not the layer of Huxley (Fig. 1E,F).

Occasionally cells in the dermis of embryonic and newborn skin were stained (see Fig. 1C) but this labeling was determined to be non-specific as it also appeared in controls.

Forestomach

The mouse forestomach is a keratinized tissue that, like epidermis, elaborates granular and cornified layers. Localization of Fos in this tissue demonstrated positive cells immediately beneath the cornifying cells (Fig. 2A,B). As with the other tissues examined, incubation of the antibody with the 25 amino acid synthetic M-peptide eliminated this immunoreactivity (Fig. 2C).

Oral epithelium

Adult rat oral epithelia displayed (Fig. 3) similar associations of Fos-positive cells with cornification. In buccal (Fig. 3A), gingival (Fig. 3A,B), tongue (Fig. 3C) and palatal (data not shown) epithelia, Fos-positive cells appeared high in the viable layers of these epithelia, just prior to cornification. Changes in Fos labelling were associated with abrupt epithelial transitions. With the transition from buccal to gingival in the adult rat oral epithelium, an enhancement of Fos labelling in the basal cell population was noted (Fig. 3A). The transition from the keratinized gingival epithelium to the nonkeratinized junctional epithelium was associated with an abrupt decrease of Fos labelling (Fig. 3A,B). While some Fos labelling is found in the nonkeratinized junctional epithelium, it was less intense and more random than labelling in keratinizing tissues. The tongue (Fig. 3C) also displayed a similar association of Fos labelling with cornification, particularly in the filiform papilla. As with all other tissues examined, competition of the antibody with the M-peptide resulted in the elimination of nuclear binding (Fig. 3D).

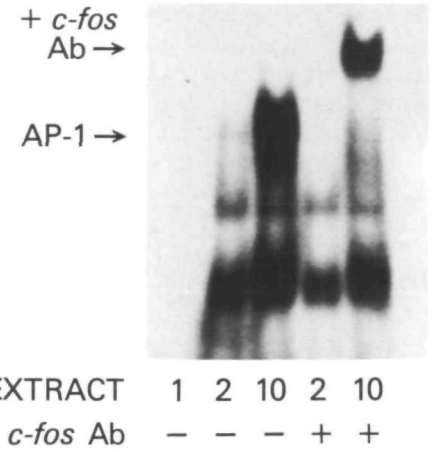

Fig. 5. Gel shift analysis demonstrates an AP-1 shift elicited by 10 μl of extract of newborn mouse epidermis. Addition of M-peptide antibodies causes a retardation in migration of the AP-1 complex (+c-*fos* AB).

Vagina

The nonkeratinized vaginal epithelium of 0 and 24 h post-estradiol-treated ovariectomized mice exhibited no detectable immunoreactivity with the M-peptide antibody. However, 72 h after administration of estradiol, the cells in the upper, viable layers of the keratinizing vaginal epithelium exhibited an intense positive reaction with the antibody (Fig. 4C,D). These Fos-positive cells were located immediately beneath a well-formed stratum corneum.

Gel retardation analysis

Extracts of newborn murine epidermis caused a marked retardation of oligonucleotides representing the GALV enhancer AP-1 binding site (Fig. 5). The M-peptide antibodies produced a further retardation of migration of the AP-1-complex (Fig. 5). Competition with cold GALV oligonucleotides completely eliminated the AP-1 shift, while a random oligonucleotide did not compete for AP-1 (data not shown).

Discussion

The data presented demonstrate a distinctive associ-

ation between the expression of Fos protein and keratinization. The process of keratinization results in the destruction of cellular organelles, and the appearance of the keratin pattern and cornified cell envelopes typical of a dead, cornified cell. This cataclysmic process is mediated by a variety of mechanisms involving the processing and assembly of keratins and keratohyalin (Dale *et al.* 1988; Fisher *et al.* 1987*a*; Bowden *et al.* 1984), and the degradation of cell organelles, presumably by catalytic enzymes including proteases and nucleases (Brody, 1959; Lavker and Matoltsy, 1970). Furthermore, this process is rapid and must be regulated so that the destructive mechanisms involved do not interfere with the assembly of the components of the cornified cell. In the light of these facts, it is anticipated that a number of genes might be activated or repressed immediately prior to entering the destructive phase of the keratinization process. We have demonstrated in neonatal and adult rodent tissue that Fos protein is expressed just prior to cornification and cell death in a variety of diverse keratinizing epithelia including epidermis and hair, oral tissues including tongue, palate, gingiva and buccal mucosa, the vagina in estrus and the forestomach. Nonkeratinized epithelia including embryonic epidermis, junctional epithelium, and the vagina in diestrus express Fos protein in reduced or undetectable levels. Our results suggest that Fos expression plays an important role in keratinization, possibly in the activation or repression of genes important to cornification. It is not clear what genes are activated or repressed in these cells but reports have suggested an important role for c-*fos* in the activation of proteases including collagenase (Schonthal *et al.* 1988) and transin (Kerr *et al.* 1988).

The transition from a viable to cornified cell is presumably rapid because cells bearing ultrastructural features of both viable and cornified cells, so-called 'transitional cells', are surprisingly rare. The Fos-positive cells that we find high in the epidermal granular layer may represent cells that are primed to undergo this transition. It is tempting to speculate that the apparent even spacing of Fos-positive cells in the upper granular layer (Fig. 1) is related to the columnar organization of cells described for mouse trunk epidermis (MacKenzie, 1969). It may be that cells are triggered to keratinize within these so-called epidermal proliferative units (Potten, 1981) in precisely the same position, possibly in the center of the column, resulting in the apparent non-random distribution of Fos-labeling.

While the labelling of cells immediately prior to keratinization with anti-Fos antibodies is a consistently reproducible finding, the association of Fos-labeling with proliferative (basal) cell populations is more variable. Labelling of interfollicular epidermal basal cells occurs in some preparations and not in others. In the hair follicle, labelling was restricted to post-mitotic cell populations. Perhaps the most informative observation on the relationship between proliferation and differentiation can be made in oral epithelia, where well-defined borders divide one epithelial type from another (Fig. 3). Differences in basal cell labelling in these tissues are often associated with the transition from one epithelium to another (Fig. 3A) These results suggest that Fos-expression in basal cell populations may have less to do with regulation of cell proliferation than with control of cell phenotype. Some caution, however, should be exercised in the interpretation of these results. Failure to identify Fos within certain populations of cells may be due to the stability of the protein or limitations in sensitivity of our technique.

The antibodies employed in the immunohistochemical studies were prepared against a 25 amino acid synthetic peptide from a conserved region of c-*fos* responsible for mediating specific DNA binding (Franza *et al.* 1987; Nakabeppu and Nathans, 1989; Quinn *et al.* 1989). The span of 25 amino acids against which the antibodies were prepared is highly conserved for both *fra-1* and *fos*-B (Zerial *et al.* 1989) and the antibodies will likely recognize any of these Fos family members. All of these Fos proteins are rapidly induced by growth factors and are capable of interacting with Jun proteins (Rauscher *et al.* 1988; Zerial *et al.* 1989) to form transacting complexes. Several lines of evidence indicate that the antibodies employed in these studies are Fos-specific: (1) the antibodies recognize AP-1 enhancer element complexes, (2) the antibodies bind primarily to the cell nucleus, and (3) elimination of antibody or competition with the Fos peptide result in the elimination of nuclear binding. As c-*fos* is rapidly induced in epidermal keratinocytes stimulated to differentiate (Dotto *et al.* 1986), we believe that c-*fos* is the major Fos protein of the intact epidermis.

The cumulative data suggest a fundamental role for Fos in regulation of late stages in the process of keratinization. Normal, keratinizing rodent tissues from diverse and different origins such as epidermis, oral epithelia, vagina and forestomach all show similar patterns of Fos expression. The primary similarity among these tissues is the appearance of Fos-positive cells just prior to cell death and cornification. Normal, nonkeratinized epithelia such as the junctional epithelium (Fig. 4), the vaginal epithelium of mice in diestrus (Fig. 5) and the embryonic epidermis (data not shown) show levels of Fos expression that are greatly reduced or undetectable by the methods employed.

The epidermis of the *Er/Er* mutant mouse fails to keratinize (Holbrook *et al.* 1982; Fisher, 1987; and Fisher *et al.* 1987*a*) and shows alterations in expression of Fos (Fig. 2D). Unlike normal, nonkeratinized epithelia, the *Er/Er* epidermis shows an abnormal expression of Fos, with virtually every nuclei staining positively. These results are enigmatic when held up against our results for Fos localization in normal tissues but we do not believe they exclude a central role for Fos in regulating keratinization. Fos expression probably represents an early step in a complex cascade of events that ultimately results in keratinization. The *Er* mutation may result in a block of this process downstream from Fos expression. Alternatively, there is evidence to suggest that the *Er* mutation acts systemically (Fisher, 1987; Fisher *et al.* 1987*a*). This

would indicate that aberrant expression of Fos in the mutant epidermis may be due to abnormal systemic delivery of factors to the mutant epidermis. The aberrant expression of Fos in the *Er/Er* epidermis may explain, in part, the abnormalities in regulation of genes for epidermal structural proteins that have been reported for this mutation (Holbrook *et al.* 1982; Fisher, 1987; Fisher *et al.* 1987*a*). These results also suggest that other elements, such as the stage of differentiation of the cell in which Fos is expressed, may be important for cornification.

The Fos family of genes are all rapidly induced by growth factors in the presence of protein synthesis inhibitors (Greenberg and Ziff, 1984; Cohen and Curran, 1988; Zerial *et al.* 1989) and participate in the formation of a DNA binding, transcription activating complex (Rauscher *et al.* 1989; Turner and Tjian, 1989; Nakabeppu and Nathans, 1989). While these genes have been primarily studied as immediate-early genes in the mitogenic response, it is well established that c-*fos* is transiently induced in cells, including epidermal keratinocytes, stimulated to differentiate (Kruijer *et al.* 1984; Dotto *et al.* 1986). The Fos-positive cells in keratinizing tissues are not only well past competence for mounting a mitogenic response but are entering the final stages of differentiation and cell death.

Our results suggest an important role for Fos in regulating the terminal step in the process of keratinization. The data presented suggest that Fos expression is a highly conserved, fundamental mechanism by which a variety of epithelial cells from diverse embryological origin may control their final stages of differentiation. In order to address this possibility, it will be necessary to identify the genes upon which Fos is acting in these terminally differentiated cells.

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