Tissue- and stratum-specific expression of the human involucrin promoter in transgenic mice
(epidermis/keratinocyte)

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ABSTRACT Involucrin is a marker of keratinocyte terminal differentiation and is expressed only in the suprabasal layers of stratified squamous epithelium. In a previous study with various cell types in culture, we noted that expression of the putative human involucrin promoter was keratinocyte specific. To determine if this promoter is sufficient to direct expression to the suprabasal cells of stratified squamous epithelium in vivo, we have now generated transgenic mouse lines harboring the involucrin promoter sequences linked to a β-galactosidase reporter gene. In the resulting lines, β-galactosidase was expressed in the suprabasal compartment of stratified squamous epithelia and in hair follicles in a tissue-specific manner. In the palate, distinct vertical stacks of β-galactosidase-expressing cells were present, suggesting movement of clonally derived cells through the epithelium. The involucrin gene has a single intron upstream of the translational start site, and removal of this intron did not affect tissue- and stratum-specific expression. These results show that the 3.7-kb involucrin upstream sequences contain all the information necessary for a high level of tissue- and stratum-specific expression.

Keratinocytes in stratified squamous epithelia exhibit specific patterns of gene expression as they undergo a progressive terminal differentiation during movement from the basal layer to the surface (1, 2). One gene expressed in suprabasal cells of this tissue encodes involucrin (3). Involucrin is a component of the cornified envelope of stratified squamous epithelia (3–5) and is a substrate, as are other envelope precursors, for transglutaminase-mediated enzymatic cross-linking (for reviews see refs. 6 and 7). In humans, the involucrin gene has been shown to consist of a short non-coding exon, a single intron, and a single exon containing the entire coding region (8). Although much is known about the function and evolution of involucrin protein (9), little is known about how the involucrin gene is regulated, other than a recent report of a phorbol 12-myristate 13-acetate-responsive element in the promoter (10).

The onset of involucrin protein expression in vitro is related to the appearance of involucrin RNA in differentiating keratinocytes (11). Although involucrin protein is detected at various levels of stratification depending on the site of tissue origin, it is invariably expressed at some point in the spinous layer (12). Unlike other markers of terminal differentiation, such as the K1/K10 keratins (13) or loricrin (14), involucrin appears to be resistant to the effects of agents which alter keratinocyte gene expression. For example, involucrin is synthesized in submerged cultures of keratinocytes (3) even though in these cultures there is no K1/K10 (15), no loricrin (16), few cornified envelopes, and no stratum corneum. Even when stratification is inhibited by low concentrations of calcium in the medium, involucrin continues to be expressed in a small fraction of cells in the basal layer (17). In raft cultures, where tissue differentiation is improved, treatment with retinoic acid suppresses both loricrin and transglutaminase expression (14, 18), whereas in the intact skin application of retinoic acid results in an apparent increase in involucrin staining (19). In healing epidermis (20), as well as psoriasis (21, 22), there is gross disruption of keratinocyte differentiation, yet involucrin expression appears prematurely but within the confines of the spinous layer. Because involucrin expression in normal epidermis is likely to be regulated at the transcriptional level (11), analysis of its promoter is likely to provide insights into why involucrin is not regulated in the same way as other markers of differentiation.

In an in vitro study of the involucrin upstream region we noted that a 3.7-kb fragment conferred keratinocyte-specific expression in transient assays (23). To determine if this 3.7-kb fragment encoded tissue and stratum specificity, transgenic mouse lines were generated with this construct linked to a β-galactosidase (β-gal) reporter gene. In the transgenic mice examined, reporter gene expression was confined to suprabasal cells of stratified squamous epithelia, and interesting patterns of expression were noted in internal epithelia.

MATERIALS AND METHODS

Plasmid Construction for the Transgenes. The construct used in this study is derived from pNAss-β-P L (23) and, as shown in Fig. 1A, includes the 3.7-kb involucrin sequences, a simian virus 40 (SV40) intron, the β-gal gene, and an SV40 poly(A) signal sequence. The HindIII site on the 5′ end represents a site 2.5 kb upstream of the involucrin transcriptional start site. A second construct lacking the involucrin intron was used and is shown in Fig. 1B.

Preparation of Transgenic Mice. The involucrin expression cassette was isolated on a 0.8% agarose (Boehringer Mannheim) gel, extracted from the gel by using Geneclean (Bio 101) purification, run through an NACS-52 Prepac column (Bethesda Research Laboratories), precipitated with ethanol, resuspended in Ca- and Mg-free phosphate-buffered saline (PBS) at a concentration of 5 μg/ml, and microinjected into mouse embryos (strain C3H-B6 F1; Harlan–Sprague–Dawley). Injections and implantations were

Abbreviations: β-gal, β-galactosidase; SV40, simian virus 40; RT, reverse transcriptase; X-Gal, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside.
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carried out by using standard protocols (24). For mouse screenings, genomic DNA was isolated from ears of 4-week-old mice and polymerase chain reaction (PCR) analysis was performed with β-gal primers. Primers to the β-gal (lacZ) gene were chosen to yield a fragment of 680 bp (upstream primer, 5'-TGGGTACACTAGTGAAGCAT; downstream primer, 5'-GATCGACAGATTTGATCCAGCGATA). DNA-positive lines were then histochemically screened for 5-bromo-4-chloro-3-indoly β-d-galactoside (X-Gal) expression in the tail epidermis (see X-Gal Histochemistry below). One founder (368-2) with intense β-gal staining was outbred to generate an F1 line which was used for all subsequent analysis.

Southern Analysis. Genomic DNA isolated from tails was digested with BamHI, which cuts twice within the transgene to yield a 4-kb band, and the resultant DNA was electrophoresed on a 1% agarose gel. After blotting to nitrocellulose, filters were incubated with a 635-bp 32P-labeled β-gal probe, washed, and exposed to X-ray film. Copy number was estimated by comparing band intensities, as measured with a laser densitometer, to known standards of the 4.0-kb β-gal fragment, and variations in loading were assessed by reprobing the blots for the single-copy β-actin gene band.

RNA Analysis. Total RNA was isolated from the organs of 4- to 10-week-old F1 mice by using RNAzolB reagent (Cinna/Biotec Laboratories, Houston), followed by digestion with DNase I and precipitation with ethanol. A reverse transcriptase (RT)-PCR kit (Perkin-Elmer/Cetus) and random primers were used to amplify 0.5 μg of total RNA to cDNA. Half of the reaction mixture was subjected to PCR using β-gal primers and half to PCR using actin primers to determine if comparable amounts of RNA were present in all tissues. The β-gal primers yielded a 680-bp band, and the actin primers yielded a 217-bp band. RNA was mock-PCR amplified (no reverse transcription) to ensure that all traces of DNA were removed.

X-Gal Histochemistry. Tissue was harvested from mice and placed overnight in a solution of 30% sucrose in PBS. The next day tissue samples were snap frozen at −70°C in O.C.T. embedding compound (Tissue-Tek, Miles) and 8-μm-thick sections were cut by using a Reichert Histostat Cryostat (model 855) and placed on gelatin-coated slides. Sections were postfixed for 10 min in 2% paraformaldehyde, washed, and stained en face with X-Gal (25). After staining in X-Gal solution for 2 hr at 37°C, slides were rinsed and counterstained by means of the Feulgen reaction (using the Schiff reagent). Nontransgenic mouse tissues were also stained to assess endogenous β-gal activity.

RESULTS

Generation of Transgenic Mice. Transgenic mice were generated with an expression vector containing the involucrin upstream sequence, an SV40 intron, the β-gal coding region, and an SV40 poly(A)+ sequence (Fig. 1A and ref. 23). Three to five weeks after birth, 35 founder mice were screened for β-gal sequences and 4 positive mice were detected. These were further screened for β-gal expression by using X-Gal histochemical staining on tail skin. Transgene copy number in the DNA-positive founders was determined by Southern analysis and was 2–50 copies per cell (Fig. 2). The intensity of X-Gal staining in tail skin did not correlate with transgene copy number. For example, line 359-2 with approximately 42 copies per cell had very light X-Gal staining, whereas line 353-6 with approximately 2 copies per cell had quite intense staining. Line 368-1, which was negative for β-gal DNA, was also negative for X-Gal staining. Mouse line 368-2 was chosen for further study as preliminary examination of tail skin showed that staining was more intense and more uniformly distributed than in the other founder mice. Line 368-2 had approximately 41 copies of the transgene per cell. To avoid the possibility of mosaic expression sometimes seen in founder mice (26), all further analysis of line 368-2 was performed on F1 offspring derived by mating with outbred strain C3H.

Tissue- and Stratum-Specific Transgene Expression. To determine the tissue distribution of transgene expression, total RNA was isolated from various tissues of mouse line 368-2 and subjected to RT-PCR amplification. Results are shown in Fig. 3. Mouse β-actin RNA served as a positive control and yielded a PCR band of 217 bp of similar intensity in all tissues. An intense 680-bp β-gal band was observed in

![Fig. 1. Depictions of transgene constructions. SalI 1 fragments of involucrin upstream sequences in plasmid pNAssβ-p.l2 are shown. The construct in A encompasses 3.7 kb of the upstream sequences and contains the involucrin intron, whereas the construct in B lacks the 1188-bp involucrin intron. Both transgenes contain the SV40 16S/19S intron and a SV40 poly(A)+ signal.](image-url)

![Fig. 2. Southern analysis of transgenic DNA from founder mice. Samples (2 μg) of genomic DNA isolated from the ears of five founder lines were digested with BamHI, electrophoresed, and blotted to nitrocellulose. The blot was probed with a 635-bp 32P-labeled β-gal probe, washed, and exposed to X-ray film. To calibrate for copy number, DNA derived from plasmid pNAssβ-p.L2 was electrophoresed at two concentrations (far right). Blots were stripped and reprobed with a mouse β-actin gene probe (not shown) representing a single-copy gene in each lane. The intensity of the bands was scanned with a laser densitometer and the intensity was compared to known copy number amounts of plasmid pNAssβ-p.L2 (after adjusting for intensity of mouse β-actin signal). The approximate copy number of the transgene was then noted in the bottom of the figure. One founder line, 368-1, was negative for β-gal DNA and is included here as a negative control.](image-url)
sequences without the involucrin intron (Fig. 1B). Of these lines, line 367-2 (containing approximately 30 copies of the transgene) exhibited the most intense staining and was bred for further analysis. It should be noted that an SV40 intron was present in the upstream region of this construct to ensure that a splicing event did take place. RT-PCR analysis of RNA extracted from an F1 derivative of the 367-2 founder indicated that line 367-2 expressed p-gal RNA was present only in line 367-2 having stratified squamous epithelia (data not shown). Histological analysis of skin and oral mucosa of line 367-2 revealed X-Gal staining confined to suprabasal cells of the epithelia (staining of buccal mucosa shown in Fig. 4F). Staining in the epidermis of line 367-2 was patchy and noticeably less intense compared with staining in line 368-2. It is evident that the involucrin intron is not required for stratum-specific expression. The reduced levels of transgene expression in mouse line 367-2 made an analysis of tissue specificity less meaningful, but when expression was detected, either by histochemistry or by RNA analysis (data not shown), it was always in stratified squamous epithelia. The involucrin intron is therefore not essential for tissue-specific expression, although its presence in the promoter appears to ensure high expression in all tissues.

**DISCUSSION**

To determine if a 3.7-kb segment of DNA upstream of the involucrin coding region was sufficient for tissue- and stratum-specific expression in vivo, this segment was linked to a p-gal reporter gene and used in the construction of transgenic mice. Expression in the resulting mice was confined to the suprabasal cells of stratified squamous epithelium, thereby demonstrating its promoter regulatory role in vivo. The involucrin promoter therefore joins with the human K5, K14, and K1 promoters, as well as the bovine K10 promoter, in being correctly expressed with both tissue and stratum specificity in stratified epithelia of transgenic mice (26, 30–32).

In human epidermis, as well as in the epidermis of transgenic mice, K14 is expressed in the basal layer, while K1 and K10 are expressed in the suprabasal cells (33). The K14 promoter has been particularly useful in exploring the pathological consequences of expression of a mutant of K14 keratin and in identifying human disease correlates of this keratin (34). Although a 12-kb fragment of the K1 promoter gives correct tissue- and stratum-specific expression in transgenic mice, it fails to respond as the endogenous mouse K1 promoter to modulators of keratinocyte differentiation, suggesting that additional sequences are needed to mediate the correct responses (31). As discussed in the introduction, involucrin is constitutively expressed during differentiation and is not subject to modulation in the same way as are other markers of differentiation. Analysis of the involucrin transgene promoter is therefore likely to lead to clues as to how these different forms of regulation are achieved.

Involucrin (35) and several other precursors of the cornified envelope that are expressed specifically in differentiating keratinocytes map to chromosome location 1q21. These proteins include loricrin [a precursor of the cornified envelope (36)], trichohyalin [an intermediate filament-associated protein of hard keratin (37)], and profilaggrin [a filament aggregating protein (38)]. The clustering of these genes at chromosomal location 1q21 is reminiscent of the globin gene cluster on human chromosome 16q22 (1). The coding regions of each of these keratinocyte genes contain a multiple repeat subunit structure and have no introns. In all cases there is an intron located upstream of the translational start site, and in the case of profilaggrin, a second intron lies further upstream in the noncoding region. The similar intron organization and the genetic linkage of these genes suggest that these introns have provided some essential function in that location to be
so preserved during evolution. The results of this study indicate that the involucrin intron is not essential for tissue- or stratum-specific expression and may serve some other function, possibly related to facilitating a high level of expression in certain tissues. The initial intron in the human keratin 18 gene is known to possess transcriptional regulatory activity through an AP-1 binding site (40). The involucrin intron contains an AP-2-like site, and such sites are required for epidermal-specific expression of keratin 14 (41). The fact that involucrin intron is required for the high level of expression in vitro (23) lends some support to the hypothesis that this intron is needed in vivo for high expression.

Stratified squamous epithelium is a tissue that undergoes continual loss by desquamation at the surface and renewal by replication of stem cells in the basal layer (42). The progeny of stem cell division, including amplifying cells and terminally differentiated cells, are organized into a spatially conserved unit known as the “epidermal proliferation unit” or EPU. In some regions of the epidermis, where keratinization is complete and turnover is slow, vertical columns of cornified and granular cells are seen and thought to be the progeny of single stem cells (28). The vertical stacks of labeled cells seen in the oral mucosa beginning in the basal layer and spanning upwards to the surface provide direct visualization of the
ordered movement of progeny through the tissue. This also marks the first time of which we are aware that an EPU-type organization has been seen in oral mucosa. The lack of a stratum corneum in this noncornifying tissue has prevented visualization of ordered columns of cornified cells, as is found in the epidermis. A similar columnar pattern has been seen in transgenic mice in which a K14-directed transgene was expressed in a mosaic fashion in the basal layer of the epidermis and the resulting transgene product was carried along in the progeny cells in the overlying spinous layer (26).

Because the involucrin promoter is faithfully expressed in all stratified squamous epithelia and is insensitive to environmental influences, it might be particularly well suited as a vehicle for keratinocyte-mediated gene therapy (43). This might be valuable for potential medical applications, including drug delivery and correction of epidermal disease states. The fact that expression from the involucrin promoter is confined to suprabasal spinous and granular cells may also allow targeting of the new gene product to specific strata while sparing the basal population of cells.

Note Added in Proof. Similar tissue- and differentiation-specific results have been obtained with the human involucrin promoter in transgenic mice. See Crish et al. (44).

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