The Distal and Proximal Regulatory Regions of the Involucrin Gene Promoter have Distinct Functions and Are Required for *In Vivo* Involucrin Expression

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Involucrin is a marker of human keratinocyte differentiation. Previous studies show that the human involucrin gene promoter has two distinct regulatory regions – the proximal regulatory region (PRR) and the distal regulatory region (DRR). To study the role of these regions *in vivo*, we have constructed human involucrin promoter transgenic mice and monitored the impact of specific promoter mutations on involucrin gene expression. In this study, we monitor the impact of specific mutations on expression in a range of surface epithelia. We begin by confirming previous observations made in footpad epidermis by showing that the full-length involucrin promoter drives differentiation-appropriate expression in other surface epithelia, including epidermis, cervix, and esophagus. We further show that mutation of the activator protein AP1-5 site in the DRR completely eliminates transgene expression in all of these tissues. In contrast, mutation of the DRR Sp1 site reduces overall expression, but does not alter the differentiation dependence. Additional studies identify a DRR immediate suprabasal element (ISE). Deletion of the ISE results in a loss of transgene expression in the immediate suprabasal layers. Our studies also indicate that the PRR is important for appropriate transgene expression. Mutation of a PRR C/EBP (CCAAT enhancer binding protein) transcription factor binding site results in patchy/discontinuous expression. These studies suggest that AP1, Sp1, and C/EBP transcription factors are required for appropriate differentiation-dependent involucrin expression, and that the mechanism of regulation is similar in most surface epithelia.

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INTRODUCTION

The epidermis is a differentiating system in which basal proliferative cells undergo discrete rounds of proliferation. The daughter cells from this division undergo profound morphology changes as they proceed through the differentiation process (Alonso and Fuchs, 2003). The ultimate fate of these cells is to be lost from the body surface after performing their protective function. These morphological changes are driven by changes in gene expression. During this process, a specific set of differentiation-appropriate genes are turned on as part of the differentiation program. Several of these genes encode proteins that are used to assemble the cornified envelope. Involucrin is a cornified envelope precursor protein (Rice and Green, 1979; Green, 1980; Watt and Green, 1981; Etoh *et al.*, 1986; Yaffe *et al.*, 1992, 1993; Eckert *et al.*, 1993; Steinert and Marekov, 1997). Involucrin is not expressed in basal cells, but expression ensues in the immediate suprabasal layer (spinous layer) and is maintained throughout the granular layer. As such, involucrin is considered an early marker of the differentiation process. A major goal of our effort is to understand the mechanisms that regulate differentiation-dependent gene expression of involucrin as a model for epidermal gene regulation.

Previous studies identified two segments within the human involucrin (hINV) promoter that are required for optimal expression in cultured human epidermal keratinocytes (Welter *et al.*, 1995; Banks *et al.*, 1998; Efimova *et al.*, 1998; Agarwal *et al.*, 1999). These elements, called the distal regulatory region (DRR) and the proximal regulatory region (PRR), are required for optimal expression in cultured cells (Welter *et al.*, 1995). The DRR contains an activator protein (AP1 site, AP1-5, and an adjacent Sp1 site that have been implicated as being required for expression in footpad epidermis (Welter *et al.*, 1995; Banks *et al.*, 1998; Crish *et al.*, 1998). The PRR contains CCAAT enhancer binding

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Abbreviations: AP, activator protein; C/EBP, CCATT enhancer binding protein; DRR, distal regulatory region; hINV, human involucrin; ISE, immediate suprabasal element; PRR, proximal regulatory region

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protein (C/EBP) and AP1 (AP1-1) sites that are required for optimal expression in cultured human epidermal keratinocytes (Welter *et al.*, 1995; Efimova *et al.*, 1998; Agarwal *et al.*, 1999). Transgenic studies indicate a role for the AP1-5 site in epidermis, as mutation of this site results in absence of expression (Crish *et al.*, 1993, 1998, 2002). In the present report, we describe transgenic mouse studies that further define the role of these elements. In particular, we monitor the effects of altering the DRR and PRR sites on expression in a range of surface epithelia. We confirm an important role for the DRR AP1-5 site and Sp1 sites. Our studies further indicate that a DNA sequence element located in the DRR segment, the immediate suprabasal element (ISE), is required for appropriate expression. In addition, the PRR C/EBP factor binding site is also required for appropriate expression. Finally, our studies suggest that hINV expression requires similar *cis*-acting DNA regulatory elements in a range of surface epithelia. This suggests that common mechanisms regulate involucrin expression in diverse epithelia.

RESULTS

The hINV DRR AP1-5 site is required for expression in diverse epithelia

We began by analyzing the expression of the full-length hINV transgene, hINV H6B. Animals that harbor the hINV H6B transgene were created and expression was analyzed by immunoblot and immunohistology. The structure of the H6B transgene is shown in Figure 1a. As shown in Figure 1b, hINV immunoreactive material is detected in the footpad, epidermis, and esophagus of hINV H6B transgenic mice. Staining of sections with anti-hINV reveals an absence of hINV protein expression in the basal layer (arrow) of footpad, cervix, epidermis, and esophagus. However, strong expression is observed in the suprabasal layers in each tissue. As expected, replacing the rabbit anti-hINV antibody with a rabbit anti-IgG as primary antibody results in an absence of detectable staining, demonstrating specificity of the reagents. In addition, staining is not observed in the underlying connective tissue layer in any tissue, indicating that expression is epithelial-specific. Staining of non-transgenic mice reveals an absence of staining with the hINV-specific antibody (not shown) (Crish *et al.*, 1998, 2002). Figure 1c shows that mutation of the AP1-5 site in H6B(AP1-5mm) results in a complete loss of expression in all epithelia, as confirmed by immunoblot and immunohistology. These studies confirm our previous reported findings in footpad epidermis (Crish *et al.*, 1993, 1998, 2002) and show that the AP1-5 site has an important role in several surface epithelia.

The isolated DRR directs appropriate expression in multiple epithelia

Previous cell culture studies implicate the AP1-5 and Sp1 sites within the DRR as being important for regulation (Welter *et al.*, 1995; Banks *et al.*, 1998, 1999; Efimova *et al.*, 1998; Bikle *et al.*, 2002). To examine the role of these elements in greater detail, we cloned the DRR immediately upstream of the hINV basal promoter to create DRR-P3.4B (Figure 2a). We have previously shown that the basal promoter transgene, P3.4B, does not drive expression in mice (Crish *et al.*, 1998, 2002) (see Figure 3b). As shown in Figure 2b, linking the isolated DRR to the hINV minimal promoter drives normal differentiation-appropriate expression. Mutation of the AP1-5 site, in the context of the isolated DRR element in DRR-P3.4B(AP1-5mm), results in a complete loss of hINV expression in all tissues (Figure 2c). We also mutated the Sp1 site, located immediate downstream of the AP1-5 site (Banks *et al.*, 1998, 1999), to create DRR-P3.4B(Sp1m). As shown in Figure 2d, mutation of the Sp1 site results in a reduction in hINV expression, as evidenced by the decreased hINV level relative to the β-actin (see immunoblot). However, although transgene expression level is reduced, appropriate suprabasal expression is maintained (Figure 2d).

Given the importance of the AP1-5 and Sp1 sites within the DRR, we examined additional DRR modifications. These constructs are shown in Figure 3a. Analysis of these transgenic mice reveals that constructs including nucleotides −2,473/−1,993 and −2,473/−2,043 produce expression patterns identical to those observed for the complete DRR (nucleotides −2,473/−1,953). In contrast, the DRR-P3.4B(−2,473/−2,097) transgene, which lacks the segment immediately downstream of the AP1-5/Sp1 sites, is expressed at low levels and expression is confined to the high suprabasal layers (ie absent in the immediate suprabasal layers) (Figure 3b). A transgene, DRR-P3.4B(−2,140/−2,088), encoding only the 52-nucleotide segment containing the AP1-5 and Sp1 sites, drives expression in a manner similar to that observed for DRR-P3.4B(−2,473/−2,097). Taken together, these results indicate that sequence elements within nucleotides −2,097/−2,043 are required, in conjunction with the AP1-5/Sp1 sites, for appropriate differentiation-dependent expression. In addition, a transgene containing the isolated downstream site(s), DRR-P3.4B(−2,097/−1,950), does not drive transcription in any tissue. The minimal promoter transgene, P3.4B, does not drive involucrin expression (Crish *et al.*, 2000).

To compare the relative level of transgene expression, we immunoblotted extracts of epidermis from individual transgenic lines using anti-hINV (Figure 4). In this experiment, as in all other experiments in this paper, all lines tested were of the F1 or subsequent generation, and transgene expression was observed to be maintained in each generation (not shown). As expected, owing to differences in integration site in the murine host genome, transgene expression is variable among individual lines for each construct. However, several general trends are apparent. First, mutation of the AP1-5 site, in the context of the full-length promoter or in the context of the DRR, uniformly results in a complete loss of transgene expression – in six hINV H6B(AP1-5mm) lines and seven DRR-P3.4B(AP1-5mm) lines. Second, mutation of the Sp1 site results in a consistent partial reduction in transgene expression – four of five DRR-P3.4B(Sp1m) lines (Figure 4a). Third, trimming of the DRR results in a reduction in expression level – four DRR-P3.4B(−2,473/−2,097) and three DRR-P3.4B(−2,140/−2,088) lines (Figure 4b). Finally, no expression is observed in six DRR-P3.4B(−2,097/−1,950) and in six P3.4B lines.

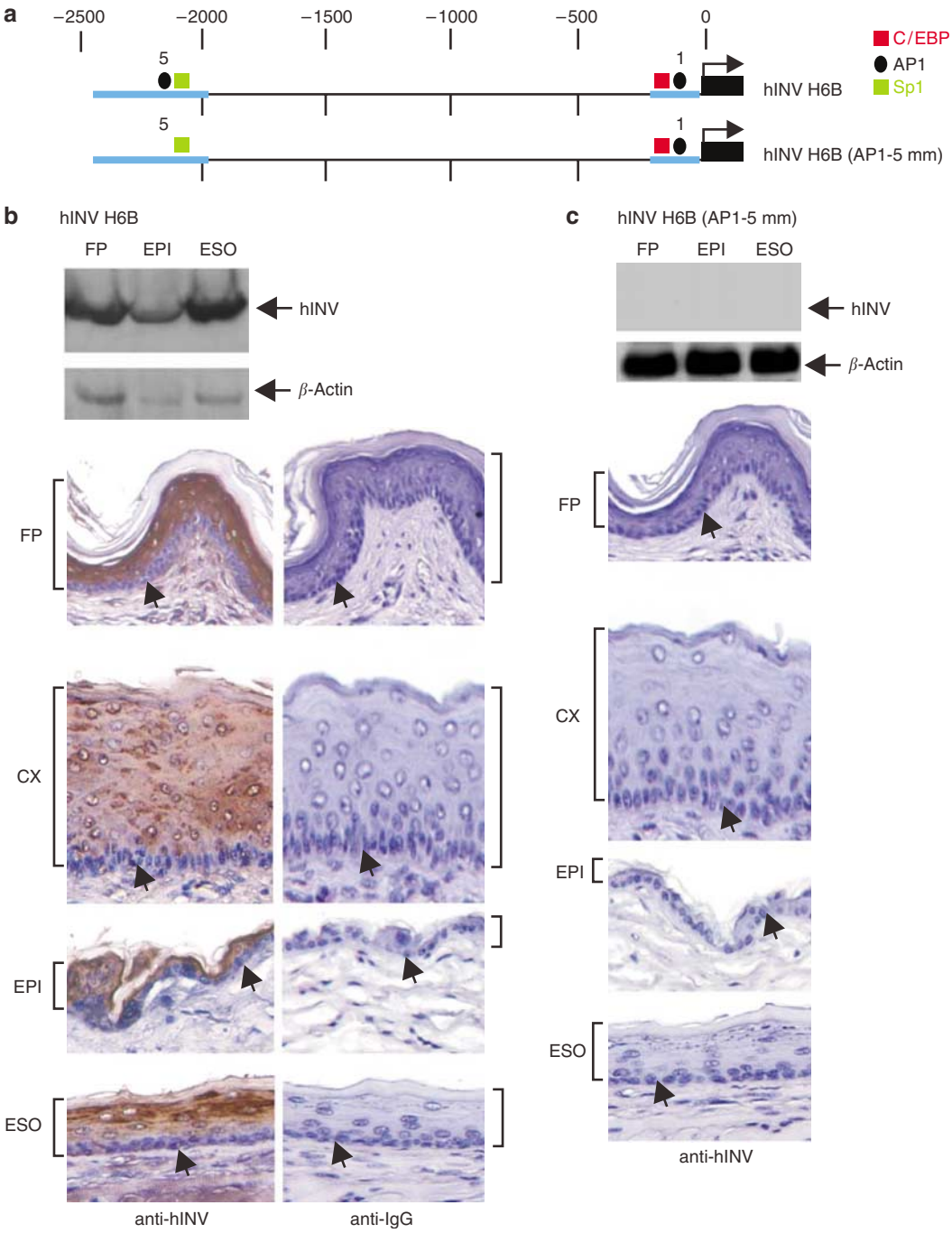

Figure 1. The AP1-5 site is essential for expression in surface epithelia. (**a**) hINV transgene structure. For each schematic, the closed box indicates the involucrin structural gene, which includes two exons separated by an intron (Eckert and Green, 1986), and the arrow indicates the transcription start site and the direction of transcription. The promoter upstream regulatory region is shown by the black line and is numbered in nucleotides upstream of the transcription start site. The PRR (nucleotides −241/−7) is indicated by the blue box adjacent to the transcription start site. Shown are the C/EBP and AP1-1 sites within the PRR. The DRR (nucleotides −2,473/−1,953), which contains the AP1-5 and Sp1 sites, is indicated by the upstream blue box. The AP1-5 site mutation, AP1-5mm, converts the AP1-5 site from 5′-TGAGTCAG to 5′-AGCTCCAG (altered nucleotides underlined) (Welter *et al.*, 1995). The mutated AP1 site is rendered non-functional (Welter *et al.*, 1995). (**b, c**) Extracts from footpad, epidermal, and esophageal epithelia were prepared in sample buffer and equivalent amounts of protein (20 μg/lane) were electrophoresed on the same gel, transferred to nitrocellulose, and incubated with rabbit anti-hINV. The blot was then stripped and reused for detection of β-actin level. Antibody binding was detected by addition of appropriate secondary antibody and visualized using chemiluminescent detection reagent. For immunohistology, sections were prepared and hINV expression was detected using rabbit anti-hINV followed by horseradish peroxidase-conjugated goat anti-rabbit IgG. The footpad (FP), cervical (CX), epidermal (EPI), and esophageal (ESO) epithelia are defined by brackets. The arrows indicate the basal layer of each epithelium. For the set of sections to the right in (**b**), anti-hINV primary antibody was substituted with rabbit anti-IgG. Secondary antibody addition and visualization are as indicated for the other sections. Similar results were observed in each of eight independent transgenic lines for each construct. The cervix was not assayed by immunoblot because the tissue is limiting.

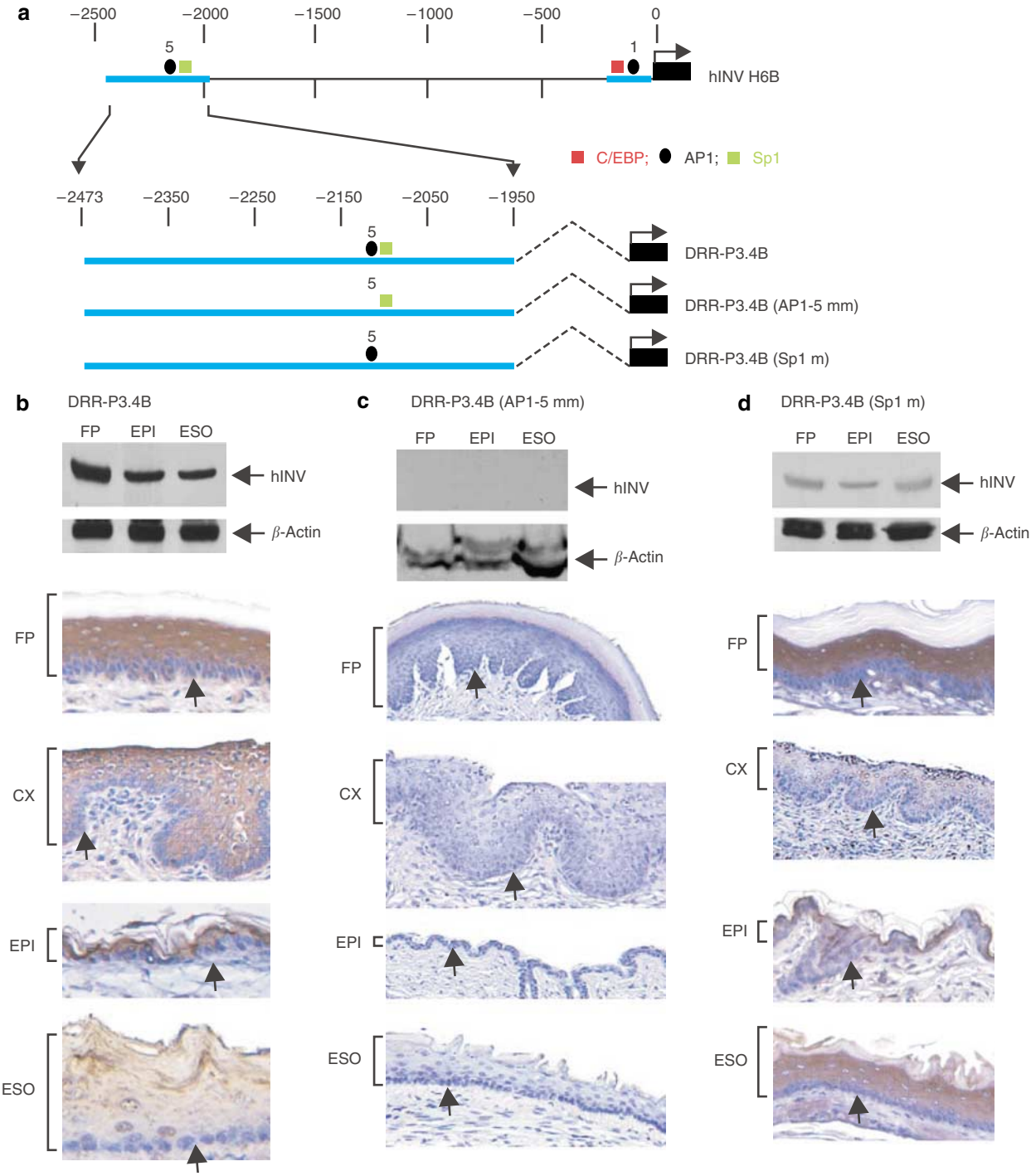

Figure 2. Role of the DRR AP1-5 and Sp1 sites. (**a**) hINV transgene structure. The hINV H6B transgene is described in the legend to Figure 1. The other transgenes encode the DRR segment linked directly to the hINV minimal promoter construct (hINV P3.4B). The dashed line indicates the point of fusion. The P3.4B minimal promoter transgene does not drive expression in any tissue (see Figure 3b) (Crish *et al.*, 1998, 2002). (**b**,**c**) The AP1-5 site mutation, AP1-5mm, converts the AP1-5 site from 5′-TGAGTCAG to 5′-AGCTCCAG (altered nucleotides underlined) (Welter *et al.*, 1995). (**d**) The Sp1 site, 5′-GGGCGGG, is mutated to 5′-GTCAAGG (mutated nucleotides underlined) in DRR-P3.4B(Sp1m) (Banks *et al.*, 1998, 1999). The mutated Sp1 site is rendered non-functional (Banks *et al.*, 1998, 1999). The remaining conventions are as outlined in the legend to Figure 1. Similar results were observed for a minimum of five independent founder lines for each transgene. It should be noted that although the intensity of signal appears to vary when measured by immunohistology, these signals are not quantitative, because the extent of peroxidase development varies. Thus, only the involucrin immunoblot signal, after normalization to the β-actin signal, accurately reflects changes in involucrin level. After scanning and normalization to the β-actin signal in each mouse strain, the level of involucrin in the DRR-P3.4B animals (five independent founder lines) was 100±12 arbitrary units as compared to 20±3 arbitrary units in DRR-P3.4B(Sp1m) mice (the F1 generation from five independent founders).

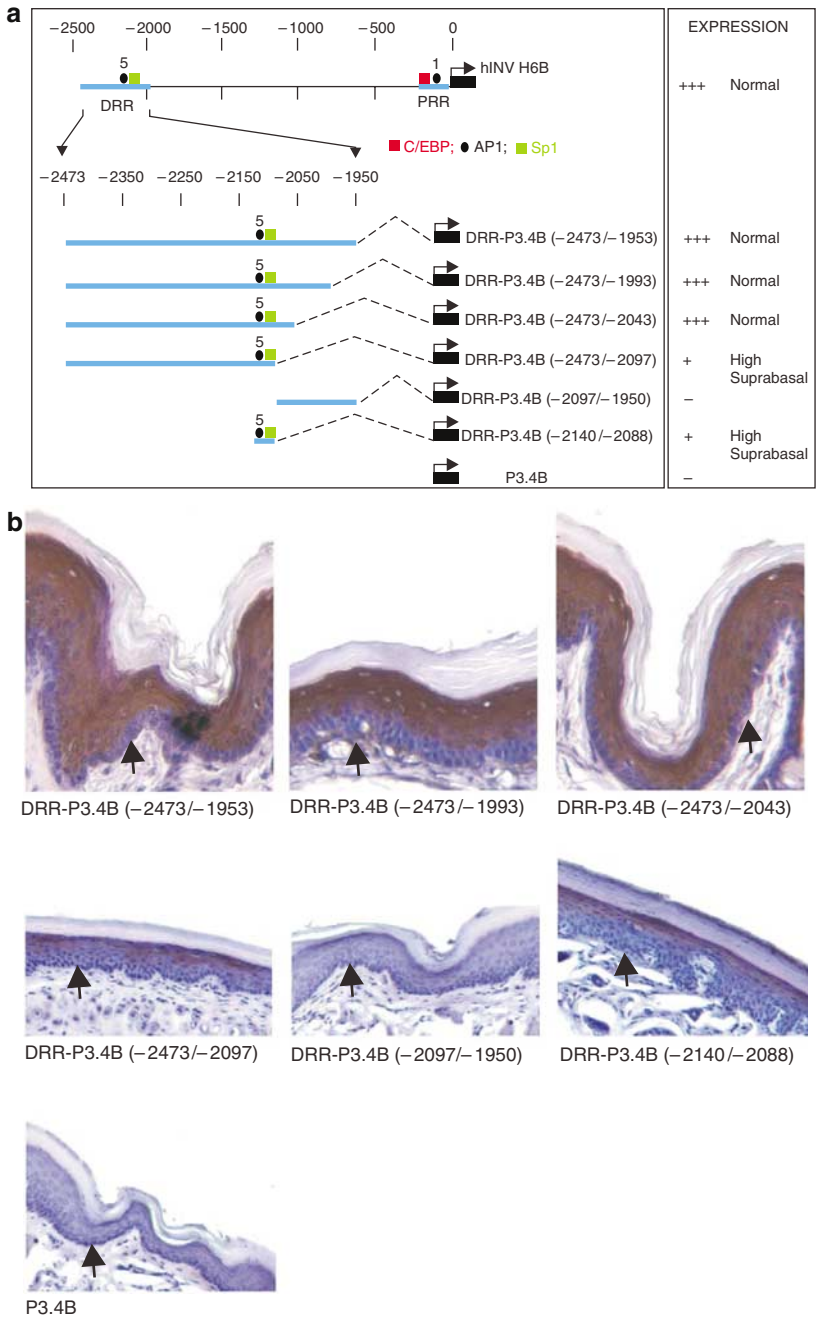

Figure 3. DRR truncation analysis – a role for AP1-5/Sp1. (**a**) The left panel shows schematics of the various hINV transgenes. H6B, the full-length transgene, is shown at the top, with the DRR and PRR segments identified. Each DRR-derived segment is linked in an identical configuration to hINV minimal promoter, P3.4B. DRR-P3.4B encodes the full-length (nucleotides −2,473/−1,953) DRR segment. The ends of the DRR-derived DNA segment present in each construct are indicated in nucleotides. The positions of the Sp1, C/EBP, and AP1-1 and AP1-5 sites are indicated. Other conventions are as in the legend to Figure 1. The right panel indicates the status of transgene expression in esophagus, cervix, epidermis, and footpad. + + + indicates optimal expression, + indicates reduced expression, − indicates absence of expression, normal indicates that the transgene is expressed in a differentiation-appropriate manner indistinguishable from expression of hINV H6B, and high suprabasal indicates that the transgene expression is confined to the high suprabasal layers. Similar results were observed in each of five independent founder-derived transgenic lines for each transgene. (**b**) Immunohistological staining of the footpad of hINV DRR truncation construct transgenic mice. Footpad sections were prepared, stained with anti-hINV and secondary antibody, and visualized as described in Materials and Methods. The arrow indicates the epidermal basal layer.

Role of the hINV PRR segment

We next examined the function of the PRR segment. This region encodes C/EBP and AP1 (AP1-1) sites that are important for promoter activity in cultured keratinocytes (Welter *et al.*, 1995; Agarwal *et al.*, 1999; Eckert *et al.*, 2004). However, the *in vivo* role of this region has not been studied. We therefore mutated the AP1-1 site and/or the C/EBP site to produce the transgenes shown in Figure 5a. Mutation of the

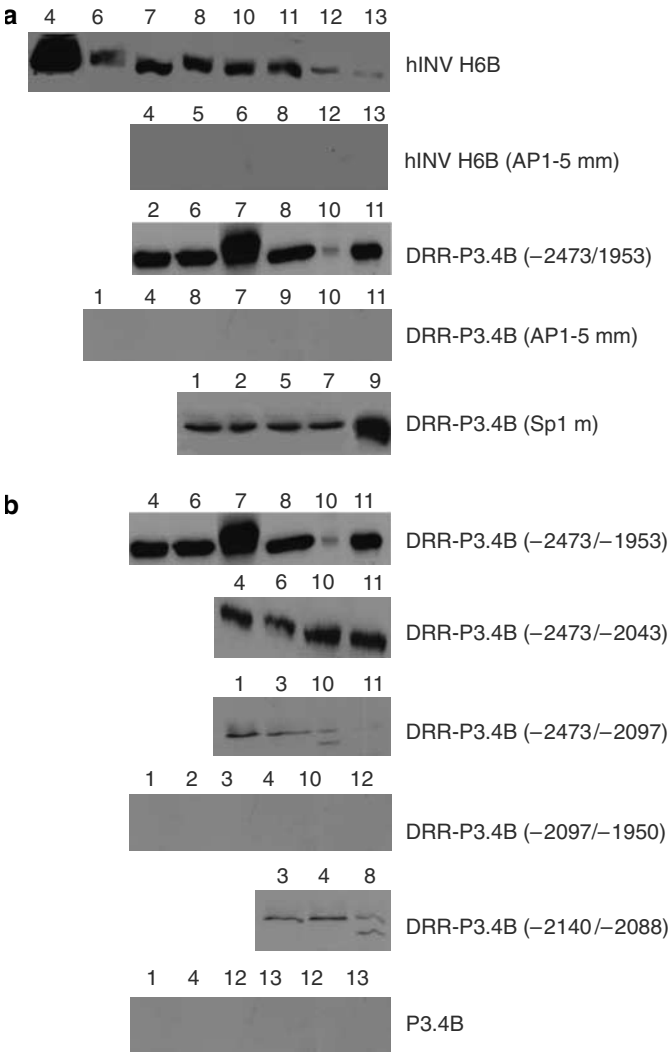

Figure 4. Involucrin transgene expression in murine epidermis. Adult mice were killed, at 3–8 months of age, and the epidermis was collected following release from the dermis by overnight treatment with dispase at 4°C. (**a**,**b**) Extracts were then prepared, electrophoresed on 6% polyacrylamide gels, and transferred to nitrocellulose for immunoblot with anti-hINV. The transgene name is indicated on the right of each blot and the number of each individual transgenic line is indicated above each lane (eg eight hINV H6B lines were tested). Each line is an independently derived strain and all animals are from the F1 or subsequent generations. Equivalent loading was confirmed by stripping the blot and incubating with anti-β-actin (not shown).

AP1-1 site results in no apparent change from the normal pattern of expression (Figure 5b). In contrast, mutation of the C/EBP site (Figure 5c) results in an atypical pattern of discontinuous expression. This phenotype is also observed in C/EBPm/AP1-1mm mice (not shown), indicating that the altered expression is due to the absence of a functional C/EBP site. This phenotype is observed in footpad and in the other tested epithelia (Figure 5c).

DISCUSSION

The process and signals that convert a basal cell to a differentiated surface epithelial cell are not well understood. Nor are the molecular events that regulate gene expression during this process. We have attempted to understand this process by focusing on the mechanisms that regulate expression of a single gene – involucrin (Eckert *et al.*, 1993, 2004). Involucrin is selectively expressed in the differentiating suprabasal cells in surface epithelia (Rice and Green, 1979; Banks-Schlegel and Green, 1981). As cells complete the final stages in terminal differentiation, involucrin is incorporated via the action of transglutaminase to form part of the crosslinked protective surface (Rice and Green, 1979; Yaffe *et al.*, 1992, 1993; Eckert *et al.*, 1993; Nemes *et al.*, 1999; Nemes and Steinert, 1999).

The hINV promoter DRR

Cell culture-based studies indicate that the AP1-5 site is required for optimal hINV promoter activity (Welter *et al.*, 1995; Banks *et al.*, 1998; Bikle *et al.*, 2002). Mutation of this site, in the context of the full-length hINV promoter, results in a partial loss of promoter activity, whereas its mutation in the context of the isolated DRR results in a complete loss of promoter activity. The finding that simultaneous mutation of AP1-5 in the DRR and AP1-1 in the PRR results in a near-complete loss of activity suggested that both these sites have important functional roles (Welter *et al.*, 1995). A role for the AP1-5 site has also been confirmed *in vivo* in mouse footpad, esophagus, and cervix, where mutation of this site results in a loss of expression (Crish *et al.*, 2002). Our present studies confirm these observations and extend these findings to the epidermis. These findings suggest that the AP1-5 site and AP1 transcription factors play a central role in maintaining hINV gene expression across a range of surface epithelial tissues. In contrast, and much to our surprise, mutation of the PRR AP1-1 site causes no change in hINV transgene expression. Thus, although AP1-1 appears to be functionally important *in vitro*, it does not appear to play a major role *in vivo*. It is possible that the AP1-1 site is not utilized owing to chromatin-mediated inaccessibility.

A role for the DRR Sp1 site

Previous studies identified an Sp1 site, located downstream and adjacent to the AP1-5 site, as being important for optimal involucrin promoter activity in cultured keratinocytes (Welter *et al.*, 1995; Banks *et al.*, 1998; Crish *et al.*, 2000). In this context, it has been suggested that Sp1 proteins may facilitate AP1 factor loading at the AP1-5 site – this is based on intracellular transcriptional regulation studies (Banks *et al.*, 1998). Indeed, our present *in vivo* studies confirm a role for the DRR Sp1 site. Its mutation results in a lower level of transgene expression. Moreover, like AP1-5, this site appears to be a universal mediator, as mutation of the Sp1 site reduces hINV expression in all tissues examined, including footpad, cervix, epidermis, and esophagus. This may be due to an impact of Sp1 interaction on AP1 factor binding to the adjacent AP1-5 site, a possibility that has been suggested (Banks *et al.*, 1998). However, it is interesting that although the Sp1 site mutation lowers overall expression, the expression remains differentiation-dependent. It is interesting that this Sp1 site is absolutely essential for involucrin expression in the corneal epithelium (Adhikary *et al.*, 2004, 2005).

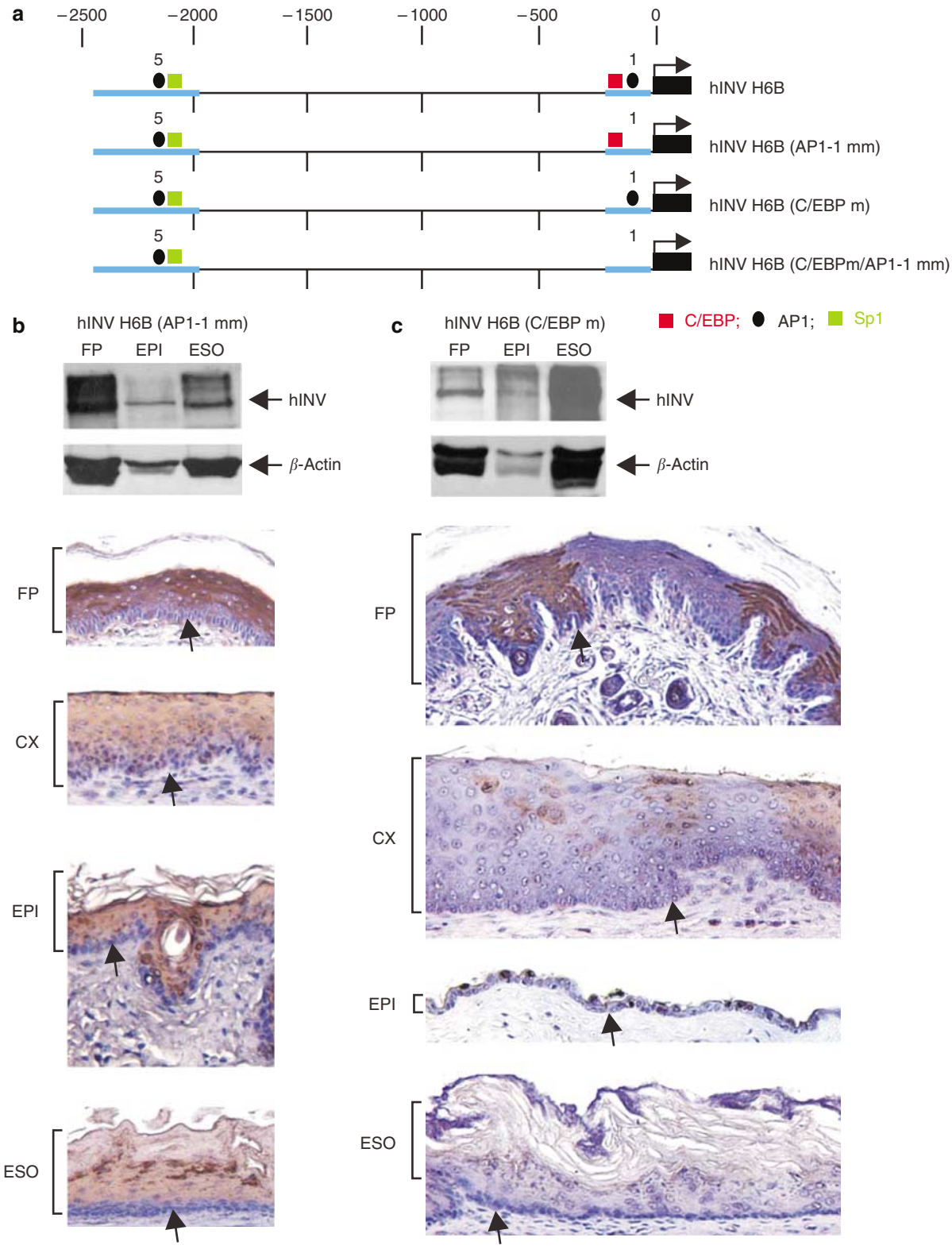

Figure 5. Role of the PRR C/EBP site. (a) hINV transgene structure. The top line shows the full-length H6B transgene. The remaining constructs show the mutants. The AP1-1, AP1-5, C/EBP, and Sp1 sites are indicated. Other conventions are indicated in the Figure 1 legend. The AP1-1 site sequence, 5′-TGAGTCAG, is mutated to 5′-AGCTCCAG (mutated nucleotides underlined) in hINV H6B(AP1-1mm). The C/EBP site, 5′-GCTGCTTAAGA, is mutated to 5′-GCTGAGATCTA (mutated nucleotides underlined) in hINV H6B(C/EBPm). The mutated sites are rendered non-functional (Welter *et al.*, 1995; Agarwal *et al.*, 1999). Both sites are mutated in H6B(C/EBPm/AP1-1mm). (**b, c**) Immunoblot and immunohistology for the hINV H6B(AP1-1mm) and hINV H6B(C/EBPm/AP1-1mm) transgene expression in mouse footpad, cervix, epidermis, and esophagus. The discontinuous pattern of involucrin expression was observed in multiple tissues of multiple transgenic F1 lines for each construct, including four hINV H6B(C/EBPm) and three hINV H6B(C/EBPm/AP1-1mm) lines.

The AP1-5/Sp1 element of the DRR

To evaluate the role of the DRR in greater detail, we tested the ability of DRR subsegments to drive gene expression. Truncation of the DRR to generate smaller and smaller segments defines multiple, spatially distinct elements as required for differentiation-appropriate gene expression. Removal of nucleotides to position −2,043 results in no change in expression as compared to the full-length DRR segment. However, removal of an additional 54 nucleotides to position −2,097 (Figure 3) results in a reduction in overall expression level and reduced expression in the immediate suprabasal layers (Figures 3 and 4). Expression is also reduced when the hINV AP1-5/Sp1 site-containing segment is tested in isolation. Moreover, expression is absent in the immediate suprabasal tissue but maintained in the high suprabasal layers. In these experiments, we chose to remove the intervening sequence when creating the DRR constructs. Although it is possible that this may change the regulatory properties of this element, we decided that this approach was preferable to inserting a large segment of spacer DNA, which may also alter regulation in an unknown manner.

The hINV promoter PRR

Promoter analysis studies in cultured keratinocytes indicate that the hINV promoter C/EBP and AP1-1 sites, located immediately upstream of the transcription start site, are required for promoter activity (Welter *et al.*, 1995; Efimova *et al.*, 1998; Balasubramanian *et al.*, 2000, 2002; Efimova and Eckert, 2000; Balasubramanian and Eckert, 2004). In the present study, we examined the effect of mutating these sites separately and together. Mutation of the AP1-1 site produces no apparent change in expression. The most remarkable change is observed following mutation of the C/EBP site. These animals display an unusual pattern of intense expression alternating with reduced or absent expression. This pattern is observed in all tested surface epithelia, but is particularly evident in the footpad. *In vitro* DNA binding assays, using extracts prepared from cultured keratinocytes, indicate that the C/EBP site binds C/EBPα (Agarwal *et al.*, 1999) and that transcription is enhanced by co-transfection of the hINV promoter with C/EBPα (Agarwal *et al.*, 1999; Balasubramanian *et al.*, 2000). Finally, the mitogen-activated protein kinase signaling cascade is known to increase C/EBPα level and binding to DNA (Efimova *et al.*, 2002). These results suggest that interfering with C/EBP factor input has dramatic effects on hINV expression.

A working model of involucrin gene regulation

Based on the available *in vivo* studies, we propose that the DRR consists of at least three key regulatory binding sites (Figure 6). The AP1-5 site is absolutely required for expression and appears to function as an on/off switch; the Sp1 site, located adjacent to the AP1-5 site, appears to function to maximize expression; and a third region, a putative ISE, appears to be required for expression in the immediate suprabasal layers (Figure 6). The PRR C/EBP site is also important, and is required for continuous expression in all tissues (Figure 6 and Table 1). Based on these findings, we propose that a transcriptional complex forms on the DRR that includes JunB, JunD, and Fra-1 at the AP1-5 site, Sp1 at the Sp1 site, and unknown differentiation-regulatory factors at the ISE (Welter *et al.*, 1995; Banks *et al.*, 1998), and that a parallel complex forms over the PRR that includes C/EBP factors (Welter *et al.*, 1995; Agarwal *et al.*, 1999),

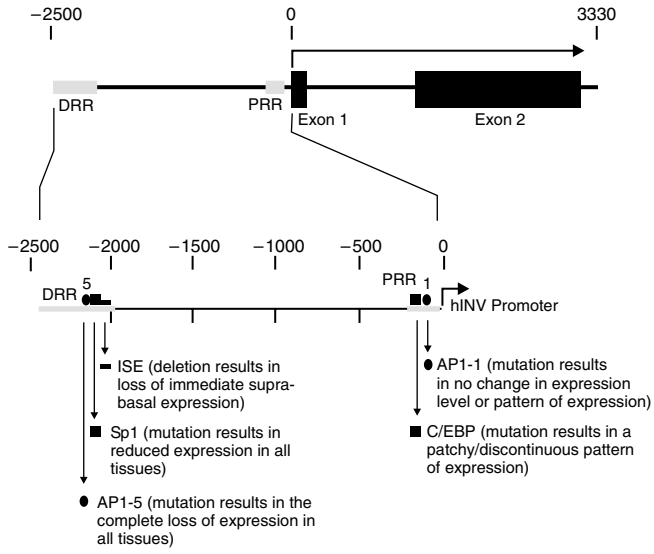

Figure 6. Spatial distribution and function of key involucrin promoter regulatory sites. The top line represents the complete involucrin gene – including the upstream regulatory region (−2,500/0) which includes the DRR and PRR, the transcription start site indicated by the arrow, and non-coding exon 1 and coding exon 2 (Eckert and Green, 1986; Crish *et al.*, 1998). The second line shows and expanded involucrin promoter upstream regulatory region. The arrow indicates the site of transcription initiation. The PRR (nucleotides −241/−7) is indicated by the gray rectangle adjacent to the transcription start site. The C/EBP and AP1-1 sites are shown within the PRR. The DRR (nucleotides −2,473/−1,953), which contains the AP1-5, Sp1, and ISE sites, is indicated by the upstream gray rectangle. The impact of mutating each element on *in vivo* involucrin promoter activity is indicated.

Table 1. Role of transcription factor binding sites in the hINV promoter

Transcription factor binding site	Location	*In vivo* function
AP1-5	DRR	Mutation results in complete loss of expression in all tissues
Sp1	DRR	Required for optimal level of expression, mutation results in reduced expression
ISE	DRR (immediately 3′ of AP1-5/Sp1 sites)	Required for expression in early differentiated layers
C/EBP	PRR	Mutation results in discontinuous expression
AP1-1	PRR	Essential for promoter activity in cultured keratinocytes - *in vivo* mutation has minimal impact on expression

and that ultimately, these factors may come into contact to fully activate differentiation-appropriate involucrin gene expression.

MATERIALS AND METHODS

Construction of hINV transgenes and production and identification of hINV gene-positive mice

The H6B transgene is a 6 kb *Hin*dIII/*Bam*HI fragment containing the complete hINV gene that was derived by restricting Charon 4AλI-3 with *Hin*dIII/*Bam*HI and subcloning the resulting 6 kb fragment into *Hin*dIII/*Bam*HI-restricted pSP64 to yield pSP64λI-3 H6B (Eckert and Green, 1986). Each of the constructs was derived from this construct using previously described methods (Crish *et al*., 1993, 1998, 2002). For microinjection, the transgenes were released from plasmid sequences by restriction with *Hin*dIII/*Bam*HI. Mouse embryos, obtained from B6CBA $\times$ B6CBA mating, were injected with each individual transgene. The injected embryos were placed into surrogate mothers (Crish *et al*., 1993, 1998) and the resulting offspring were characterized for transgene presence by hINV tail DNA blotting. The hINV cDNA probe does not crossreact with the endogenous mouse involucrin gene; thus, the presence of hINV gene sequences can be directly detected (Crish *et al*., 1993). A minimum of three and a maximum of eight individual transgenic founders were characterized for each transgene tested, and all lines were bred to the F1 and subsequent generations prior to testing. All the experiments were approved by the Institutional Animal Care and Use Committee.

Detection of hINV transgene expression

It is important to note that all of the transgenes utilized in the present study retain the hINV coding sequence in a native configuration with respect to the hINV promoter segments. To assay for transgene expression, we monitored the presence of hINV protein by immunoblot and by immunohistology. Our antibodies readily distinguish the hINV protein produced from the transgene from the endogenous murine involucrin protein (LaCelle *et al*., 1998). To detect hINV expression in mouse tissues, total protein extracts were prepared from epidermal samples following release of the epidermis with dispase treatment. The samples were then prepared in Laemmli sample buffer, and equivalent amounts of protein from each sample were electrophoresed on an acrylamide gel and transferred to nitrocellulose for antibody-dependent detection of hINV. The blot was incubated with a primary antibody prepared against recombinant hINV (LaCelle *et al*., 1998) diluted 1:600 and visualized using a chemiluminescent detection system (Crish *et al*., 1993). The blot was then stripped and incubated for detection of β-actin as a loading normalization control. For immunohistology, sections were prepared as previously described (Crish *et al*., 1993, 1998) and then incubated with rabbit anti-hINV followed by a peroxidase-conjugated goat anti-rabbit IgG and visualization by peroxidase substrate deposition.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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