

# Targeted Ablation of the Murine Involucrin Gene

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**Abstract.** Involucrin is synthesized in abundance during terminal differentiation of keratinocytes. Involucrin is a substrate for transglutaminase and one of the precursors of the cross-linked envelopes present in the corneocytes of the epidermis and other stratified squamous epithelia. These envelopes make an important contribution to the physical resistance of the epidermis. We have generated mice lacking involucrin from embryonic stem cells whose involucrin gene had been ab-

lated by homologous recombination. These mice developed normally, possessed apparently normal epidermis and hair follicles, and made cornified envelopes that could not be distinguished from those of wild-type mice. No compensatory increase of mRNA for other envelope precursors was observed.

**Key words:** embryonic stem cells • envelopes • skin morphology • skin resistance • involucrin

## Introduction

The protective function of the mammalian epidermis depends largely on the presence of the chemically resistant envelope of the corneocytes. The envelope consists of proteins stabilized by  $\epsilon(\gamma\text{-glutamyl})\text{lysine}$  cross-links formed under the action of transglutaminase 1, an enzyme specific to keratinocytes (Rice and Green, 1978; Simon and Green, 1985; Thacher and Rice, 1985). The protein envelope is covered with a monomolecular layer of *N*-( $\omega$ -hydroxyacyl)sphingosine bound to protein by ester bonds (Swartzendruber et al., 1987; Wertz and Downing, 1987; Marekov and Steinert, 1998).

Involucrin is synthesized in the outer living layers of terminally differentiating keratinocytes of all stratified squamous epithelia (Banks-Schlegel and Green, 1981). Human involucrin has been shown to be a preferred substrate of keratinocyte transglutaminase and a precursor of the cross-linked envelope (Rice and Green, 1979; Eckert et al., 1993; Yaffe et al., 1993). Expression of human involucrin in transgenic mice results in abnormalities of the skin and hair (Crish et al., 1993).

The nucleotide sequence of the involucrin gene has been determined in a large number of anthropoid and

nonanthropoid mammals. In all species examined, the coding region of the gene contains a segment of short tandem repeats, which accounts for one half to two thirds of the coding region. The entire involucrin molecule, and particularly its segment of repeats, contains numerous glutamine residues (Eckert and Green, 1986), clearly relating to the function of the protein as an amine acceptor in transglutaminase-catalyzed cross-linking (Tseng and Green, 1988, 1990; Phillips et al., 1990, 1997; Djian and Green, 1991; Djian et al., 1993).

Other precursors of the cross-linked envelope are loricrin (Mehrel et al., 1990), the small proline-rich proteins (SPRRs)<sup>1</sup> (Kartasova and van de Putte, 1988), and two membrane-bound proteins, periplakin and envoplakin (Simon and Green, 1984; Ruhrberg et al., 1996, 1997). Recently, additional envelope constituents including S100 proteins have been described (Robinson et al., 1997). Whereas the role of transglutaminase 1 in the synthesis of the cornified envelope is clear (Jeon et al., 1998), the relative importance of the various substrates has not been established. Therefore, we decided to generate mice lacking involucrin by targeted ablation of the gene. Surprisingly, the absence of involucrin had no obvious effect on the cornified envelope, the morphology of the epidermis, or the hair follicles.

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<sup>1</sup>Abbreviations used in this paper: ES, embryonic stem; SPRR, small proline-rich protein.

## Materials and Methods

### Construction of the Targeting Vector

The whole involucrin coding region, which is confined to a single exon, was replaced with a neomycin phosphotransferase gene. We had isolated previously the involucrin genes of the NIH Swiss and BALB/c mice (Djian et al., 1993). To transform J1 embryonic stem (ES) cells, which are derived from a male agouti 129/SV mouse (Li et al., 1992), with isogenic DNA, we isolated the involucrin gene of a 129/SV mouse genomic library in the  $\lambda$ FIXII vector (Stratagene). Screening of  $10^6$  phage DNA plaques (Sambrook et al., 1989) with a 1.2-kb HindIII fragment containing most of the involucrin coding region identified two positive clones, of which clone  $\lambda$ 17B was used to prepare the targeting vector. This clone contained the coding region flanked by 1.5 kb of upstream sequence and 11.5 kb of downstream sequence. The entire insert of  $\lambda$ 17B was excised with NotI (whose site is present in the  $\lambda$ FIXII vector, on both sides of the insert) and subcloned into pBluescript. The resulting plasmid (psv6) was cut with NotI, XbaI, and PstI to excise most of the coding region and to subclone the flanking sequences. These sequences were isolated as a 1.7-kb NotI–PstI fragment (upstream) and a 10.6-kb XbaI–NotI fragment (downstream), thus generating psv18 and psv26, respectively. Since psv18 retained 95 codons, including the initiator ATG, and since we were not certain that the encoded peptide could not act as a substrate in transglutaminase-catalyzed cross-linking, we deleted those codons with BAL31. The resulting clones were sequenced and one clone (psv23) was used in subsequent experiments; it lacked the entire coding region, the 3' splice site, and a small 3' part of the intron (see Fig. 1). The neomycin phosphotransferase gene under the control of the phosphoglucokinase promoter and followed by the phosphoglucokinase 3' end, including the polyA addition site, was excised from pGK-neo (a gift from Dr. Fred Alt, Children's Hospital, Boston, MA) with EcoRI and HindIII. An XhoI site was added immediately 3' of the HindIII site by subcloning the EcoRI–HindIII fragment into pBluescript and re-excising it with EcoRI and XhoI. The resulting fragment was cloned directly into the linker of psv23, in the right orientation and downstream of the involucrin 5' sequence. The insert of the resulting plasmid was excised with NotI and XhoI, blunt-ended with the Klenow fragment, and cloned with XbaI linkers upstream of the XbaI–NotI insert of psv26. The sequence flanking the XbaI site of the linkers was such as to regenerate the NotI site that had been partially abolished by treatment with the Klenow enzyme. The orientation of the XbaI insert in the clones obtained was determined, and one of the constructs, psv29, was used to transfect ES cells. To facilitate homologous recombination, we excised the insert with NotI and removed the pieces of linker on both ends by a brief treatment with BAL31, before transfection of the ES cells.

### Transfection and Selection of ES Cells

The J1 ES cell line was cultivated on a feeder layer of irradiated embryonic fibroblasts (6,000 rads). The fibroblasts were prepared from day 14

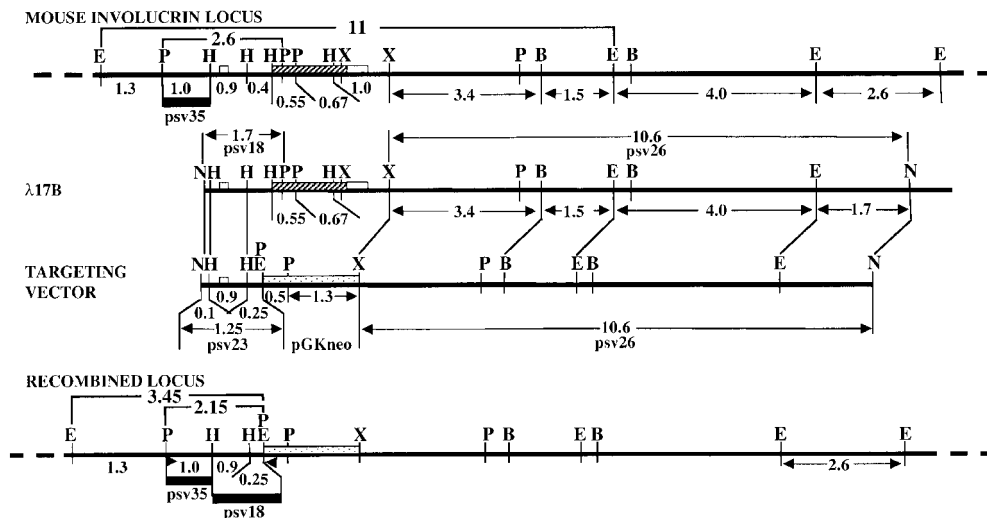
mouse embryos homozygous for the neomycin resistance gene. ES cells were grown in bicarbonate buffered DMEM supplemented with 15% FCS (heat inactivated at 56°C for 30 min), nonessential amino acids, 500 U/ml of murine leukemia inhibitory factor (ESGRO; GIBCO BRL), penicillin, streptomycin, and 0.11 mM 2-mercaptoethanol. For electroporation of DNA, ES cells were trypsinized, washed with electroporation buffer, and resuspended at  $2 \times 10^7$  cells/ml. The cells were mixed with 120  $\mu$ g of DNA and electroporated at 0.4 kV and 25  $\mu$ F, at room temperature, using a gene pulser apparatus (Bio-Rad Laboratories). After electroporation, the cells were cooled on ice for 10 min, and then plated on a feeder layer. After 24 h, the transfectants were selected in the presence of 0.4 mg/ml of G418 (GIBCO BRL). After 9 d of selection, individual drug-resistant clones were picked and transferred to 96-well plates. When the cells were about half confluent, they were trypsinized; half was left in the well and frozen at  $-120^\circ\text{C}$  in the presence of 20% DMSO, whereas the remaining half was transferred to 24-well plates, for screening by Southern blotting. Positive clones were injected into BALB/c and C57BL/6 blastocysts at the Brigham and Women's Hospital Core Transgenic Mouse Facility, Boston, MA (by Dr. Arlene Sharpe).

### Southern Blot Analysis

DNA was prepared from ES cells or mouse tails by lysis in the presence of 100 mM Tris-HCl, pH 8.5, 200 mM NaCl, 5 mM EDTA, 0.2% SDS, and 0.1 mg/ml proteinase K. The lysate was incubated at 55°C for at least 6 h, and the DNA was precipitated in the presence of isopropanol, spooled out, and resuspended in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA (Laird et al., 1991). Southern blots were performed as described in Tseng and Green (1988). The probe was a 1.0-kb PstI–HindIII fragment located immediately 5' to the genomic fragment used to prepare the targeting vector (see Fig. 1). To clone the probe, we first constructed a library from size-fractionated genomic DNA. After digestion with PstI and electrophoresis of the digest, fragments of  $\sim 2.6$  kb were excised from the agarose gel and ligated to pBluescript. The ampicillin-resistant bacteria were divided into 22 pools, each containing  $\sim 7,000$  primary transformants. Screening by Southern blotting with the 1.25-kb NotI–PstI fragment located immediately upstream of the involucrin coding region revealed two positive pools. The fragment of interest was amplified by PCR, using an upstream primer located in the plasmid, and a downstream primer corresponding to codons 2–8 of the involucrin gene. The PCR product was cut with PstI and HindIII and the mixture of fragments was ligated directly into pGEM3z cut with PstI and HindIII. All recombinants obtained contained the fragment of interest (psv35).

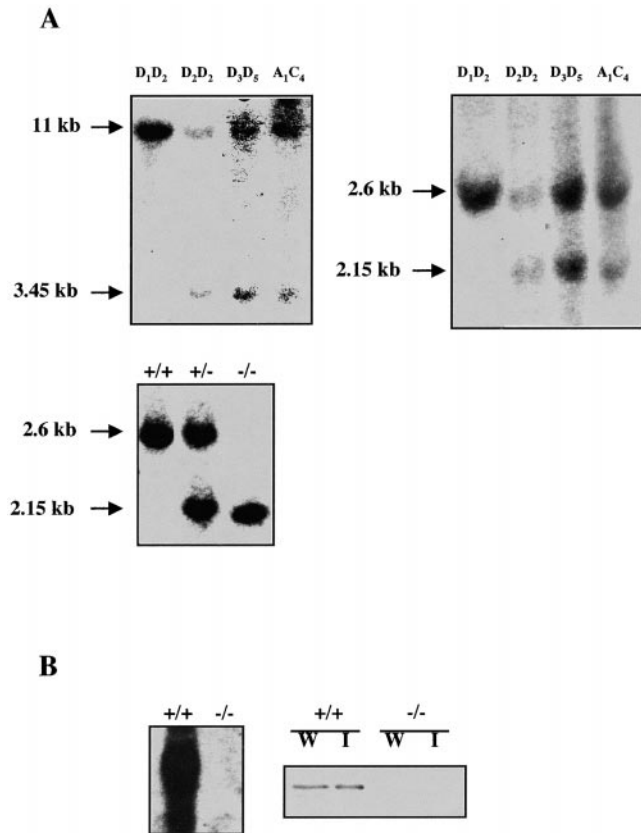
### Northern Blot Analysis

For preparation of total skin RNA, mice were first anesthetized by intraperitoneal injection of tribromoethanol (Sigma-Aldrich). Hairy mice were depilated with a commercial preparation containing thioglycolic acid. The skin was then removed, frozen immediately in liquid nitrogen, pulverized in



**Figure 1.** Ablation of the involucrin gene. Diagram of the involucrin locus, the replacement construct, and the resulting recombined locus. Distances between restriction sites are given in kilobases. The three boxes represent the involucrin coding region (stippled), the neomycin phosphotransferase gene (dotted), and the probes (psv35 and psv18) used for screening ES cells and mice (solid). psv35 used in conjunction with either EcoRI or PstI identifies unambiguously the recombined locus, since it hybridizes to specific fragments of 3.45 kb

(EcoRI) and 2.15 kb (PstI); the corresponding fragments derived from the natural locus are 11.0 and 2.6 kb, respectively. Psv 18, which hybridizes to the construct itself, was used to demonstrate that transgenic mice did not contain additional copies of the construct inserted by nonhomologous recombination. E, EcoRI; H, HindIII; N, NotI; P, PstI; X, XbaI.



**Figure 2.** Southern, Northern, and Western blot analysis of wild-type mice and mice lacking involucrin. (A) Southern hybridization with psv35 of genomic DNA prepared from ES clones (top) or mouse tails (bottom). Screening of DNA prepared from neomycin-resistant clones and digested with either EcoR1 (top left) or Pst1 (top right) identified three positive clones giving rise to 3.45- and 2.15-kb fragments ( $D_2D_2$ ,  $D_3D_5$ , and  $A_1C_4$ ); one negative clone is also shown ( $D_1D_2$ ). Clone  $A_1C_4$  was used to generate chimeric males, which were backcrossed to produce heterozygous progeny. Heterozygotes were then intercrossed, and the progeny were genotyped with Pst1. One wild-type (+/+), one heterozygote (+/-), and one  $inv^{-/-}$  mouse (all littermates) are shown in the bottom panel. (B) Northern and Western blot analyses of skin prepared from a wild-type and an  $inv^{-/-}$  mouse. The  $inv^{-/-}$  mouse contains neither detectable involucrin mRNA (left) nor the corresponding protein (right). W, whole protein extract; I, partially purified involucrin.

a mortar, resuspended in Tripure isolation reagent, and purified according to the manufacturer's recommendation (Boehringer). Total RNA was analyzed by electrophoresis in agarose gels containing formaldehyde, according to Sambrook et al. (1989), and RNA was transferred to nylon without prior treatment of the gel. Prehybridization, hybridization, and washing conditions were exactly as those described for Southern blotting. The probes consisted of a 1.2-kb fragment containing most of the involucrin coding region (Djian et al., 1993), a 0.9-kb BamHI-PstI fragment containing the 3' half of the loricrin coding region (Mehrel et al., 1990), and a 0.7-kb EcoR1 fragment derived from the SPRR1 cDNA (Kartasova et al., 1996).

### Western Blot Analysis

Protein extracts containing involucrin were prepared from mouse skin. Pulverized skin was prepared as described above for RNA. Approximately 400 mg of tissue was resuspended in 2 ml of a buffer containing 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 2 mM EDTA, 0.1 mM leupeptin, 0.01 mM pepstatin, and 1 mM PMSF. The tissue was homogenized (Potter Elvehjem), clarified by two centrifugations at 120 g for 15 min and 48,000 g for 30 min, and the supernatant was collected. The protein concentration of the supernatant was determined with a protein assay kit using IgG as

standards (Bio-Rad Laboratories). Protein extracts were stored at 4°C in the presence of 1% SDS. Partially purified mouse involucrin was prepared according to Etoh et al. (1986).

The proteins were resolved by SDS-PAGE, using 4 and 6% acrylamide plus bisacrylamide for the stacking and resolving gels, respectively (a ratio of 29:1 for both stacking and resolving gel). Immunoblot analysis was carried out according to Kahlem et al. (1998). The antiinvolucrin (Djian et al., 1993) and the secondary antibody were both added at a 1:4,000 dilution.

### Histological Analysis and Preparation of Cornified Envelopes

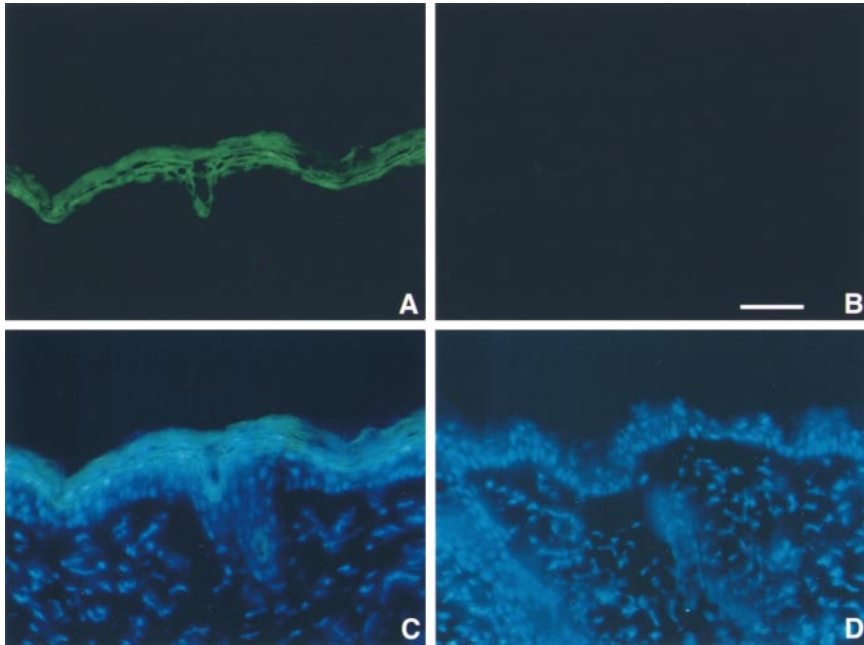
For sections stained with hematoxylin and eosin, the skin samples were first fixed in 10% buffered formalin and embedded in paraffin then stained. For immunofluorescence studies of involucrin, skin samples were snap frozen in OCT (Miles) and isopentane and cut with a cryomicrotome at 5 mm. Sections were fixed in acetone/methanol (1:1) at -20°C and stained with rabbit anti-mouse involucrin (Covance), then were detected with goat antiserum to rabbit IgG coupled to Alexa 488 (Molecular Probes). DNA was stained using Vectashield mounting medium containing DAPI (Vector Laboratories).

For preparation of envelopes, the tip of the ear was cut, placed in water containing 25 mM DTT and 2% SDS, heated to 100°C for 15 min, and centrifuged. The pellet was resuspended in 10 mM Tris-HCl, pH 8.0, and 1 mM EDTA. Envelopes were examined in a hemocytometer under phase microscopy.

## Results

### Ablation of the Involucrin Gene

The entire coding region of the involucrin gene was replaced with the neomycin phosphotransferase gene. ES cells were transformed with the construct and neomycin-resistant clones were screened by Southern analysis, using psv35 as probe (Fig. 1). Analysis of 297 neomycin-resistant clones disclosed 3 in which the involucrin gene had been ablated by homologous recombination (Fig. 2 A). All three clones ( $D_2D_2$ ,  $D_3D_5$ , and  $A_1C_4$ ) were injected into C57BL/6 and BALB/c blastocysts to generate chimeric animals. Chimeric males derived from clone  $A_1C_4$  were backcrossed with either C57BL/6 or BALB/c females; both backcrosses yielded progeny of which 70–80% were agouti. As expected, probing of a Pst1 digest of genomic DNA with psv35 showed that agouti mice contained one ablated allele (2.15-kb fragment) and one normal allele (2.6-kb fragment) (Fig. 2 A). Reprobing of the same blot with psv18, which hybridizes to the construct itself (Fig. 1), showed that heterozygous mice contained no additional copy of a transgene that might have inserted randomly by nonhomologous recombination. Heterozygous mice were intercrossed to generate mice homozygous for the mutation. Absence of the involucrin coding region in the  $inv^{-/-}$  mice was confirmed by Southern blotting. Absence of involucrin mRNA and protein in the skin was confirmed by Northern and Western blot analyses (Fig. 2 B). Because nude mice have a thicker epidermis and more involucrin than hairy mice, we transferred the ablated allele to a nude background.  $nu/nu$  males (BALB/c) were crossed with  $inv^{-/-}$  females (C57BL/6), thereby generating F1 progeny that were  $inv^{+/-}$  and  $nu/+$ . The F2 progeny of F1 intercrosses yielded  $nu/nu$ ,  $inv^{-/-}$  at the expected frequency of 1:16. Frozen sections of mouse skin ( $nu/nu$ ,  $inv^{-/-}$  and  $nu/nu$ ,  $inv^{+/+}$ ) were stained with a rabbit antiserum prepared against recombinant involucrin (Covance). In the  $inv^{+/+}$  mice, involucrin had the typical distribution in the outer third to half of the living layers, in the stratum corneum, and in the hair follicles; no involucrin was detected in the mice lacking the involucrin gene (Fig. 3).



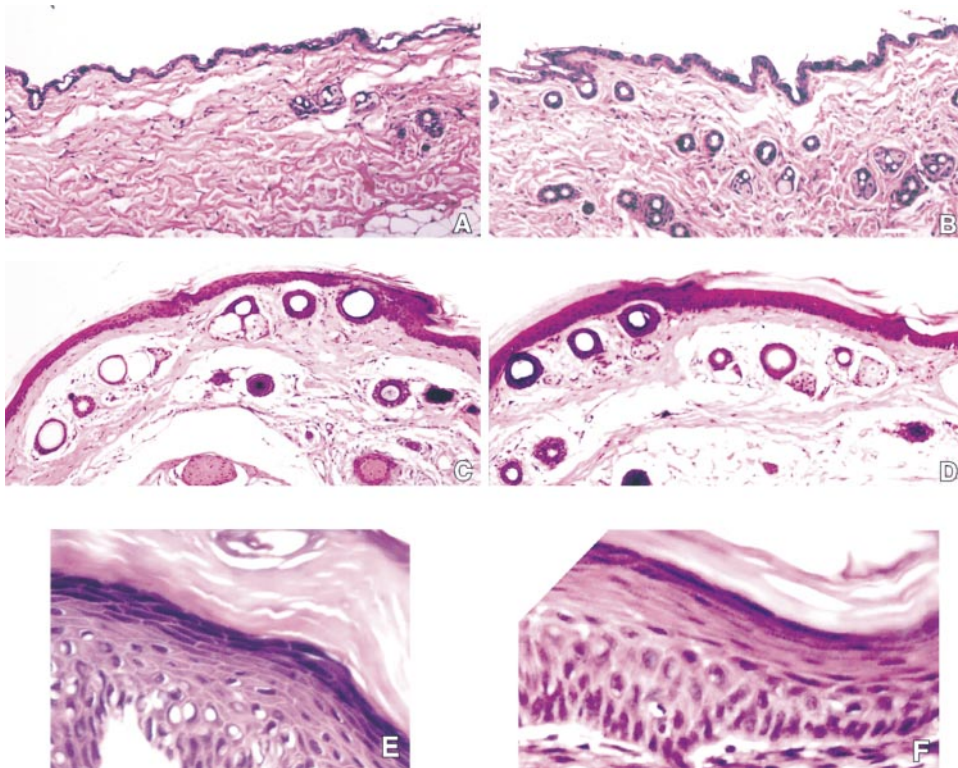
**Figure 3.** Absence of involucrin from epidermis of mice lacking involucrin by immunofluorescence. Frozen sections of back skin of nude mice (*nu/nu*, *inv*<sup>+/+</sup> and *nu/nu*, *inv*<sup>-/-</sup>) were reacted with a rabbit antiserum prepared against the recombinant protein. The secondary antibody was conjugated to Alexa 488. (A) In the *inv*<sup>+/+</sup> mice, the epidermis is brightly stained for involucrin in the outer living layers, the stratum corneum, and the hair follicles. (B) The epidermis of *inv*<sup>-/-</sup> mice reveals no involucrin staining. (C and D) The addition of the nuclear stain DAPI reveals the nucleated cells of *inv*<sup>+/+</sup> and *inv*<sup>-/-</sup>. Bar, 50  $\mu$ m.

### ***Mice Lacking Involucrin Develop and Reproduce Normally***

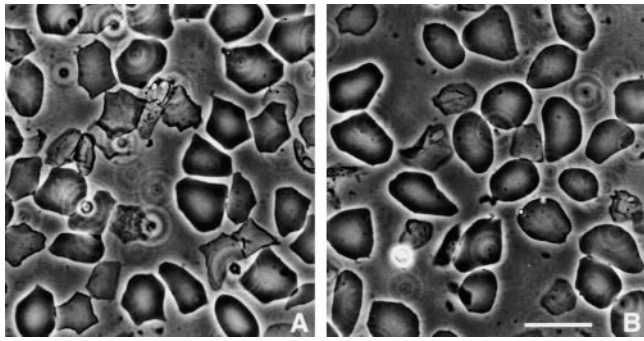
Whether produced from backcrosses with C57BL/6 or BALB/c, the *inv*<sup>-/-</sup> mice appeared normal and showed no obvious difference from their heterozygous or wild-type littermates. *Inv*<sup>-/-</sup> females intercrossed with *inv*<sup>-/-</sup> males produced litters of normal size. Among the 177 offspring examined from the heterozygous intercrosses, the numbers of wild-type, heterozygous, and *inv*<sup>-/-</sup> mice were 54 (30.5%), 87 (49.2%), and 36 (20.3%), respectively, or close to the expected Mendelian ratio of 0.25:0.5:0.25.

### ***The Epidermis and Hair Follicles of Mice Lacking Involucrin Appear Histologically Normal***

Histological sections of the back skin of adult wild-type and mice lacking involucrin were compared. No differences were observed either in the epidermis or in the hair follicles (Fig. 4, A and B). As mouse epidermis of the trunk is very thin and contains only two to three layers, we also examined epidermis of the tail, whose thickness is comparable with that of the human epidermis, and which possesses clearly distinguishable basal, spinous, granular, and cornified layers. Neither in the spinous and granular



**Figure 4.** Histological analysis of skin of mice lacking involucrin. Paraffin sections of back skin whose epidermis contains only two to three cell layers (A and B) and tail skin (C–F) of C57BL/6 mice were stained with hematoxylin and eosin. Skin of the tail contains many more cell layers and includes clearly identifiable basal, spinous, granular, and cornified layers. The absence of involucrin (B, D, and F) does not result in detectable abnormalities in either site. Bar, 50  $\mu$ m.



**Figure 5.** Cornified cell envelopes of mice lacking involucrin. Envelopes were prepared from ear skin incubated in the presence of SDS and DTT at 100°C. (A) *inv*<sup>-/-</sup>. (B) *inv*<sup>+/+</sup>. There is no clear difference between the two. The ratio of angular to balloon forms was not consistently different. Bar, 50 μm.

layers, where free involucrin is found, nor in the stratum corneum, where the involucrin is cross-linked in the envelopes, was there a perceptible difference between wild-type (Fig. 4, C and E) and *inv*<sup>-/-</sup> (Fig. 4, D and F) littermates. The healing time of the wounds resulting from the skin biopsies used for histological analysis was compared in mice lacking involucrin and normal mice and was not found to be significantly longer in the former. The gross appearance of the esophageal, conjunctival, and corneal epithelia, which also contain involucrin, appeared unchanged in its absence.

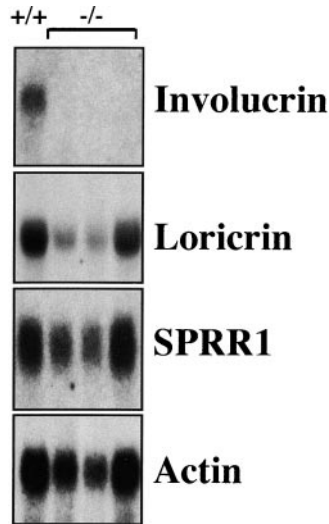
#### **Mice Form Normal Cornified Envelopes in the Absence of Involucrin**

Envelopes were prepared by removing the tips of ears of *inv*<sup>-/-</sup> and wild-type mice, heating them to 100°C for 10 min in the presence of 2% SDS and 25 mM DTT, and examining the envelopes by phase-contrast microscopy. The envelopes prepared from *inv*<sup>-/-</sup> mice were indistinguishable from those of wild-type mice (Fig. 5). Both strains of mice possessed a mixture of balloon-shaped and angular envelopes. Balloon-shaped envelopes probably originate from the outermost corneocytes of the epidermis, which have lost their desmosomes, whereas angular envelope are likely to have originated from more internal corneocytes, which were still attached to each other by the remains of desmosomes.

We compared the resistance of wild-type envelopes and envelopes lacking involucrin to various physical and chemical agents. We tested the effects of sonication, detergents (SDS, guanidine, and urea), proteases (trypsin and pronase), and grinding with a motor-driven homogenizer. Although all of these treatments caused progressive disruption of the envelopes, we could detect no difference in the resistance of wild-type envelopes and envelopes lacking involucrin.

#### **Absence of Involucrin Is Not Compensated by Increased Synthesis of Other Envelope Precursors**

In view of the lack of any detectable phenotype produced by the absence of involucrin, we thought that increasing levels of other envelope precursor proteins might have compensated for the absence of involucrin. The levels of the mRNAs encoding loricrin (Mehrel et al., 1990) and SPRR1 (Kartasova and van de Putte, 1988; Kartasova et al., 1996) were found to be identical in the mice lacking in-



**Figure 6.** Northern blot analysis showing envelope precursor mRNA levels in mice lacking involucrin and wild-type mice. Absence of involucrin is not compensated by increased levels of the mRNAs encoding SPRR1 or loricrin.

volucrin and in the wild-type mice (Fig. 6). Therefore, the absence of involucrin is not compensated by increased levels of either loricrin or SPRR1 mRNAs.

#### **Discussion**

Mutations in the human transglutaminase 1 gene result in lamellar ichthyosis (Huber et al., 1995) due to the absence of cross-linked envelopes (Jeon et al., 1998). Targeted disruption of the transglutaminase 1 gene in mice also causes the absence of envelopes, a severe disruption in barrier function, and early lethality (Matsuki et al., 1998). Therefore, it is clear that the cornified envelope is essential for the protective function of the epidermis, and that only transglutaminase 1 is essential for the synthesis of the envelope, since the other two transglutaminases present in keratinocytes (types 2 and 3) do not compensate for its absence.

A large body of data supported the earlier conclusion that involucrin was an essential component of the cross-linked envelope. The synthesis of involucrin is tightly regulated: it is restricted to terminally differentiated keratinocytes of stratified squamous epithelia. Human involucrin is a preferred substrate of transglutaminase 1, since after incubation of a crude extract of cultured epidermal cells in the presence of labeled putrescine and transglutaminase 1, involucrin was labeled at least 80 times more intensely than the average of the other cytosolic protein (Simon and Green, 1985). Involucrin was also shown to promote the cross-linking of the particulate fraction containing membrane proteins. In other respects, involucrin is thought suitable as a protein able to cross-link other proteins (Yaffe et al., 1992). Involucrin is rich in glutamine residues (Djian et al., 1993; Eckert and Green, 1986), a property in keeping with its function as an amine acceptor in transglutaminase-catalyzed cross-linking; other envelope precursors, such as loricrin, envoplakin, periplakin, or the small proline-rich proteins, are not glutamine-rich. For all of these reasons, it is surprising that envelopes should form normally in mice lacking involucrin. The presence of

these cross-linked envelopes in *inv*<sup>-/-</sup> mice clearly establishes that contrary to earlier suggestions (Steinert and Marekov, 1997), whatever properties might be conferred on envelopes by involucrin, this protein is not essential for the assembly of envelopes, which in its absence consist exclusively of other proteins.

It remains to be seen whether more detailed examination of the skin of *inv*<sup>-/-</sup> mice will reveal a detectable difference from that of wild-type mice. We have provided the *inv*<sup>-/-</sup> mice to Dr. Peter Elias (University of California at San Francisco, San Francisco, CA) and to Drs. M. Jensen and E. Proksch (University of Kiel, Kiel, Germany) for detailed examination of the water vapor barrier and ultrastructural studies of the envelopes.

The disruption of the loricrin gene, which encodes another envelope precursor (Mehrel et al., 1990), also does not prevent envelope assembly, although it results in transitory redness of skin immediately after birth (Koch et al., 2000, this issue). It seems that whereas the transglutaminase is required in order to make the cross-linked envelope, neither involucrin nor loricrin is individually required. Mutations in the COOH-terminal part of human loricrin cause Vohwinkel syndrome, but the mutated alleles are dominant and the disease is likely to result from a gain of function (Maestrini et al., 1996).

The involucrin gene of the mouse is very polymorphic and this in itself would be compatible with the absence of strong selective pressure. However, this polymorphism includes the addition of numerous repeats that increase the size of the protein from 448 to 695 residues (Delhomme and Djian, 2000). The involucrins of the BALB/c and C57BL/6 strains used in this study contain 448 and 468 residues, respectively. It is therefore necessary to suppose either that the increases in size occur in the absence of any demonstrated necessity for the protein, or that evolutionary development of a larger involucrin will eventually render the protein indispensable.

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