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***RESEARCH ARTICLE***

# *Durable and stratum-specific gene expression in epidermis*

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*A number of genetic disorders are manifested in cutaneous epithelium and gene therapy approaches for treatment of such diseases are being considered. A successful gene therapy protocol requires durable and correctly targeted gene expression within the tissue. The continuous renewal and high levels of compartmentalization in epidermis are two challenges for a successful gene therapy of skin disorders. For those disorders which affect the upper layers of epidermis, vectors must be available that target stem cells, but remain silent until the progeny of these cells undergo differentiation. To explore the potential of long-term and targeted vector expression in epidermis, a hybrid retroviral vector encoding the reporter enhanced green fluorescent protein (EGFP) was constructed. The viral enhancer in the long terminal repeat of the vector was replaced with a 510-bp enhancer element of the human involucrin promoter. Keratinocyte-specific expression directed by the hybrid vector was demonstrated in culture and suprabasal-specific expression was observed in organotypic human epidermal cultures. In vivo transduction of mouse skin with this hybrid vector indicated long-term and stratum-specific expression of the transgene in mouse epidermis. The design of similar vectors for various gene therapy applications constitutes an important step toward clinically effective gene therapy.*
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**Keywords:** *epidermis; retroviral vectors; gene therapy; targeted expression*

## *Introduction*

Gene therapy strategies are being considered for a variety of cutaneous disorders for which the genetic basis has been identified.[1] However, two challenges in developing such a therapy are sustained expression and correct targeting of the therapeutic gene. Epidermis is a highly compartmentalized tissue that is continuously renewed through proliferation of keratinocyte stem cells. Epidermal stem cells are located among their progeny of proliferating keratinocytes in the basal layer of epidermis and are the ultimate target of gene delivery. As the stem cell progeny leave the basement membrane, they withdraw from the cell cycle and undergo a regulated program of terminal differentiation involving morphological and biochemical changes. The outcome of this program is the assembly of stratified epithelium consisting of basal, spinous, granular and cornified layers.[2] The morphological and biochemical differences between these layers are manifested in genetic disorders of the epidermis. For correction of epidermal disorders that affect a specific epidermal compartment, it may be necessary to restrict transgene expression to the affected compartment. In the two models of monogenic skin disorders tested to date, lamellar ichthyosis and junctional epidermolysis bullosa, such stratum-specific expression was not required to achieve morphological correction. However, in these two instances expression of the therapeutic gene was lost over a period of several weeks.[3] Although gene inactivation is one possible explanation for this loss of expression, another possibility is a growth disadvantage to transduced stem cells and gradual loss of these cells from the tissue.[4]

Retroviral vectors are widely used to integrate and express exogenous genes into a variety of cells and provide an efficient gene transfer tool for human gene therapy applications.[5] These vectors have been used with considerable success for transduction of epidermal stem cells in culture and *in vivo*. However, transcription from the long terminal repeat (LTR) is dependent on viral promoter/enhancer elements located in the U3 region of the 5′LTR, which allow constitutive expression of the transferred gene in most cell types, including keratinocytes.[6,7] Several strategies have been employed to confer tissue- or cell-specific expression to retroviral vectors. These include insertion of a tissue-specific promoter in an internal position within the retroviral vectors, construction of self-inactivating vectors, in which viral enhancer elements are deleted thereby allowing expression from the internal promoter, and insertion of a complete minigene into the LTR upstream from the U3 region.[8] These strategies have usually resulted in decreased viral titer and have often failed to induce strict tissue-specific expression.[9] Attempts to redirect LTR transcriptional activity by replacing the viral enhancer with heterologous control elements from cellular genes have been partially successful, allowing transgene expression

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in a specific tissue or cell context without significant loss in the viral titer. However, the size of tissue-specific enhancers remains as the major limitation of this approach.[10,11]

The possibility of targeting retroviral vectors in a stratum-specific manner to the epidermis was examined using the upstream regulatory sequences of the human involucrin (hINV) gene. Involucrin is one of the precursor proteins used in assembling the keratinocyte-cornified envelope, and is specifically expressed in suprabasal layers of stratifying squamous epithelia.[12] A 2500-base fragment of hINV gene upstream regulatory region is sufficient to drive tissue- and stratum-specific expression in transgenic mice.[13,14] Deletion mapping studies have identified two regulatory regions that contribute to differentiation-induced activity of this promoter; a distal element (−2473/−2088) containing AP-1 and Sp1 binding sites, and a proximal region (−185/−1) with a critical AP-1 binding site overlapping with a consensus binding site for ets transcription factor.[15–17] We have explored the potential of these critical regulatory sequences of the hINV to restrict retrovirus-driven expression of the transgene to the suprabasal layers of the epidermis.

## Results

### *Construction of minimal transcriptional elements of involucrin promoter and analysis in transgenic mice*

Cell-specific gene expression has been reported when the retroviral enhancer in the LTR is replaced with a heterologous enhancer. However, significant reductions in the viral titers are observed when the inserted sequences are much larger than the deleted viral sequences.[10] To avoid such reduction in titer, while still achieving targeted expression in the upper strata of epidermis, essential involucrin enhancer elements were identified by analysis in transgenic mice. In an earlier study, a 2500-bp fragment of the hINV upstream region was shown to confer tissue- and stratum-specific expression (Figure 1).[14] In this fragment, consensus SP1 and AP1 binding sites are located primarily at the 5′ and 3′ termini of this region while the central region is devoid of transcription binding sites.[17] A 754-bp fragment was derived from this upstream regulatory region including AP1-5, SP1 and AP1-1 binding sites by deletion of the central *Bgl*II (−2100)/*Msc*I (−192) 1908-bp fragment. When the 754-bp fragment was used to drive LacZ expression in transgenic mice, tissue- and stratum-specific expression was evident, similar to that observed for the full-length promoter (Figure 1).[14]

### *Construction of retroviral vectors targeted to the upper strata of the epidermis*

To engineer a hybrid INV/LTR, the sequence preceding the involucrin TATA box from 754-bp truncated promoter (510-bp) was fused with viral CAAT and TATA box replacing the viral enhancer (direct repeats between *Nhe*I and *Xba*I restriction sites) in the U3 region of LTR. To assess control basal viral promoter activity, the viral enhancer was deleted between *Nhe*I and *Xba*I restriction sites to generate enhancer-deleted LTR (dLTR). The reconfigured LTR was cloned into the 3′ position of the parental retroviral vector plasmid LZRS-EGFP. Upon transduction and integration, the hybrid 3′ LTR forms the template for the 5′LTR in the proviral DNA, and directs transcription of reporter gene EGFP in target cells (Figure 2a). Producer cell lines were established by transfecting 293GP retroviral packaging cells[8] and isolating puromycin-resistant cells. Pseudotyped retroviruses were generated by transiently transfecting the producer lines with a plasmid DNA encoding vesicular somatitis virus G protein. The viral titer was assayed by transducing keratinocytes and counting EGFP+ cells by FACS analysis 48 h after transduction. The viral titer ranged between 0.5 and $2 \times 10^6$ GFP-transducing units/ml indicating that the modifications in the 3′LTR did not result in a significant drop in the titer.

To exclude possible rearrangements in the viral genome as a result of the recombination events between the wild type 5′LTR and the heterologous 3′ LTR, RNA isolated from recombinant retroviruses or from transduced keratinocytes was analyzed by RT-PCR using a set of primers flanking the U3 enhancer element in LTR. As shown in Figure 2b, when either virus RNA or total RNA from transduced cells was analyzed, the expected fragments of 460-bp for LZRS-GFP, 205-bp for dLTR-GFP and 725-bp for INV/LTR-GFP were generated (lanes 2–7). The INV/LTR-GFP plasmid DNA was used as a control to demonstrate simultaneous amplification of both 5′ LTR and 3′ hybrid LTR when both are present (lane 8). The sequence of amplified fragments was confirmed by restriction analysis using *Xba*I (lanes 11–13).

This analysis demonstrated that packaging of the viral particles and integration into the target genome were not affected by the LTR modifications, and ruled out the possibility of contamination of modified viral stock with viruses containing wild-type LTR.

### *Keratinocyte-specific expression of the hybrid retroviral vector in culture*

Keratinocyte-specific gene expression from the hybrid INV/LTR vector was assessed in several transduced epidermal and non-epidermal cell lines including, SW480 (colon-epithelial cell), NIH-3T3 (fibroblast), C2C12 (myoblast), HepG2 (hepatocytes), adult epidermal keratinocytes (KC) and newborn foreskin keratinocytes (HFKC). Approximately 50 000 cells were transduced with 50 μl of LZRS-GFP or INV/LTR-GFP or 100 μl dLTR-GFP vectors (at the multiplicity of infection of ~1). Cells were passaged once and EGFP expression was examined by FACS analysis. Transduction efficiency was determined by the percentage of EGFP+ cells and ranged between 60 and 85% as indicated in Figure 3. The median fluorescent intensity of EGFP was indicative of the level of EGFP expression. The wild-type LTR was active in all cell types examined, although the level of activity varied significantly between cell types. Interestingly, the wild-type LTR was most active in keratinocytes when compared with other cell types examined and activity of the hybrid promoter was about 50% that of the wild type. However, expression of the hybrid INV/LTR promoter was specific for epidermal keratinocytes. In all other cell types, including simple epithelial cells (SW-480), transcriptional activity of hybrid INV/LTR promoter was similar to that of enhancer-deleted LTR. These data indicated that replacement of the viral enhancer with a 510-bp enhancer element from hINV promoter conferred cell specificity to the retroviral vectors without an apparent loss in the viral titer.

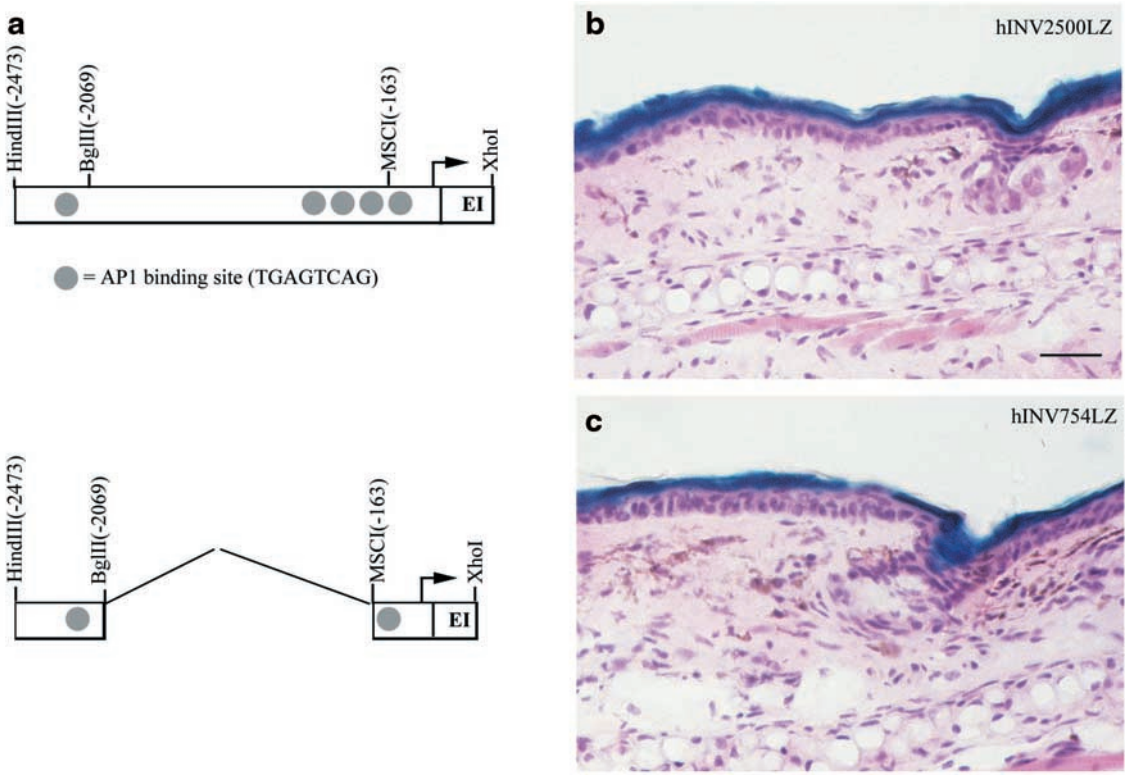


***Figure 1*** *Analysis of minimal involucrin enhancer/promoter in transgenic mice. (a) Schematic representation of the full-length and truncated hINV promoter. The position of AP-1 binding sites in the hINV promoter and the deleted sequences is indicated. (b, c) Tissue- and stratum-specific LacZ expression directed by either promoter in transgenic mice. Confinement of β-galactosidase activity (blue) to suprabasal cells of epidermis is shown in cryosections obtained from ear skin. The bar represents 50 μm.*

### *Stratum-specific expression of EGFP in epidermal raft cultures*

Involucrin expression is normally confined to keratinocytes undergoing terminal differentiation.[19] In cultures of human keratinocytes, involucrin is expressed by all cells that have left the basal layer.[12] To determine if transgene expression is confined to the upper strata of epidermis, organotypic cultures were constructed with keratinocytes transduced with retroviral vectors in which EGFP expression was directed by either the LTR or the INV/LTR hybrid promoter. Mature differentiated cultures were fixed and cryosectioned and expression of EGFP was examined by fluorescent microscopy. To examine co-localization of the endogenous human involucrin with EGFP, some sections were stained for involucrin expression using anti-human involucrin antibody. As shown in Figure 4 (c and d), involucrin expression in the organotypic cultures was epibasal (red), as described for stratified cultures or hyperproliferative human skin.[12] EGFP expression (green) was observed in all layers of cultures seeded with LTR-EGFP-transduced keratinocytes indicating pan-epithelial expression of EGFP (Figure 4a and c). However, INV/LTR-directed EGFP expression co-localized with endogenous involucrin expression to suprabasal layers of the cultures (Figure 4b and d). These data demonstrate that retroviral vectors containing the hybrid INV/LTR promoter were able to direct expression to the upper strata of the epidermis.

### *In vivo transduction of mouse dorsal skin with INV/LTR-EGFP retroviral vector*

To examine durability of transgene expression, pseudotyped LZRS-EGFP and INV/LTR-EGFP retroviral vectors were concentrated and injected into the dorsal skin of mice as described previously.[20] Repeated examination of cornified cells by tape stripping showed persistent green fluorescing cells for both LZRS-EGFP and INV/LTR-EGFP. At 20 weeks after transduction, when mouse skin has turned over at least 20 times,[21] biopsies were obtained from the transduced areas, fixed and cryosectioned. Expression of EGFP in tissue sections was analyzed by fluorescent microscopy. In normal mouse skin, expression of involucrin is observed in the upper spinous and granular layers of interfollicular epidermis.[22] As demonstrated in Figure 5 (a–c), EGFP expression was detected in both LZRS-EGFP and INV/LTR-EGFP-transduced skin indicating persistent gene expression, hence transduction of stem cells. However, the pattern of EGFP expression differed between the two groups of mice. EGFP expression directed by the viral promoter in LTR was detected in all layers of transduced epidermis and occasionally in dermal fibroblasts (Figure 5a). On the contrary, in those mice transduced with retroviral vectors containing hybrid INV/LTR promoter, EGFP expression was mainly localized to the suprabasal layers of epidermis, where the endogenous involucrin is expressed (Figure 5b–c). However, in about 5% of the transduced

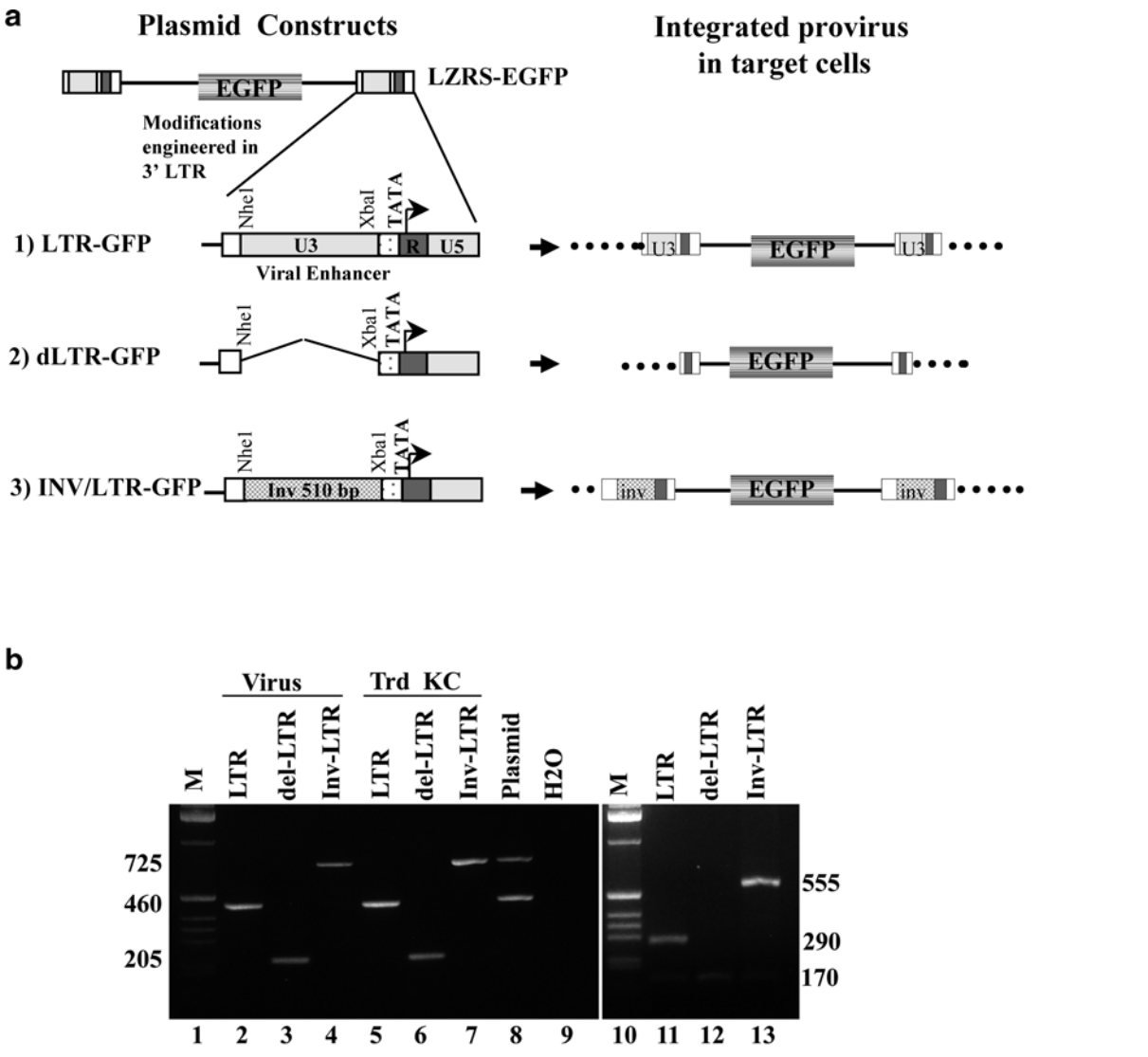


***Figure 2*** *Construction and analysis of retroviral vectors. (a) Schematic representation of retroviral vector constructs and modifications made to the 3′ LTR of the parental retroviral vector LZRS-EGFP. (1) LTR-EGFP in which expression of EGFP is driven by wild-type LTR promoter; (2) dLTR-EGFP, which incorporates a deletion of the U3 enhancer element (NheI–XbaI restriction sites) in the 3′ LTR; and (3) hybrid LTR in which the U3 enhancer is replaced by a 510-bp truncated involucrin enhancer element. The structure of the retroviral vector plasmids is shown on the left and the structure of the integrated proviral genome is depicted on the right side of the figure. (b) The viral RNA from the various recombinant virus stocks as indicated on the top of the figure (lanes 2–4) or total cellular RNA from keratinocytes transduced with these vectors (lanes 5–7) were isolated and analyzed by RT-PCR using a set of primers flanking the U3 enhancer element in LTR. In lane 8, the retroviral plasmid construct with the INV/LTR hybrid promoter in the 3′LTR was used as the template for PCR. The fragments were purified and digested with XbaI to verify the sequences as shown in the right panel. The size of amplified fragments is indicated on both sides of the figure.*

epidermal clusters ($n$ = 60), EGFP expression was observed in the both basal and suprabasal cells (data not shown). The integration of retroviral vectors in or near an active chromatin structure may be the likely explanation for this observation.[23]

EGFP expression at 20 weeks after transduction indicates that the hybrid vector has been incorporated stably into the genome of progenitor cells in the basal layer of epidermis, but remains silent until activation of the differentiation program in epidermis. These data suggest that long-term transgene expression in the compartment devoid of stem cells is possible by transcriptional targeting.

## Discussion

For long-term gene therapies of continuously renewing tissues like epidermis, stem cells are the ultimate targets of gene transfer. Recombinant retroviral vectors are highly efficient at gene transfer and gene integration into epidermal stem cells. However, compartmentalization of epidermis and its continuous renewal present a challenge for using these vectors for targeted transgene expression. Gene transfer to progenitor cells in the basal layer of epidermis results in expression of the genes in both basal keratinocytes and their suprabasal differentiated progeny, and transfer of the gene to differentiated cells in

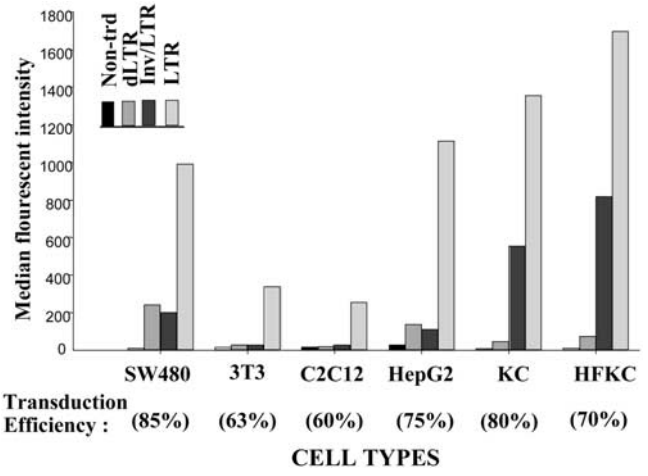


***Figure 3*** *Keratinocyte-specific expression of INV/LTR hybrid promoter in culture. Several cell lines including SW480 (simple epithelia), NIH-3T3 (fibroblast), C2C12 (myoblast), HepG2 (hepatocytes), adult epidermal keratinocytes (KC) and foreskin keratinocytes (HFKC) were transduced with each of the viruses as indicated in the figure. EGFP expression was examined by flow cytometry. Transduction efficiency was determined by FACS-EGFP analysis and ranged between 60 and 90% as indicated in the bottom of the figure. The median fluorescent intensity of EGFP was indicative of the level of EGFP expression directed by each promoter.*

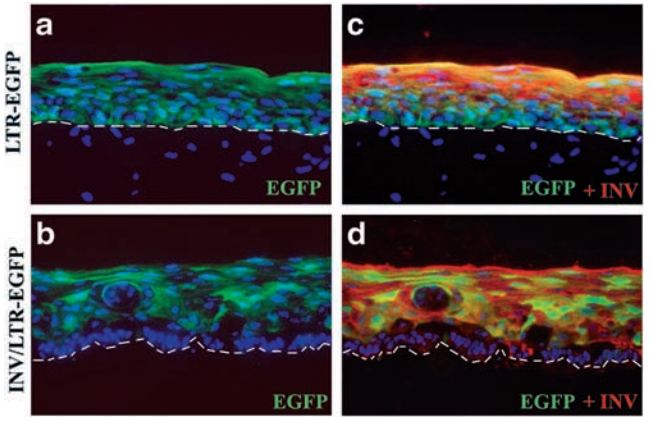


***Figure 4*** *Stratum-specific expression of EGFP in epidermal raft cultures. Three-dimensional raft cultures of human keratinocytes were constructed using keratinocytes transduced with retroviral vectors in which expression of EGFP (green) was directed by either the viral promoter in the LTR (a and c) or the INV/LTR hybrid (b and d) promoter. Rafts were fixed, cryopreserved and sections were analyzed for green fluorescent (green) or stained for human involucrin (c, d) using indirect immunofluorescent staining (red) and counterstained with DAPI (blue). The position of basement membrane is indicated with a dotted line. The bar represents 50 μm.*

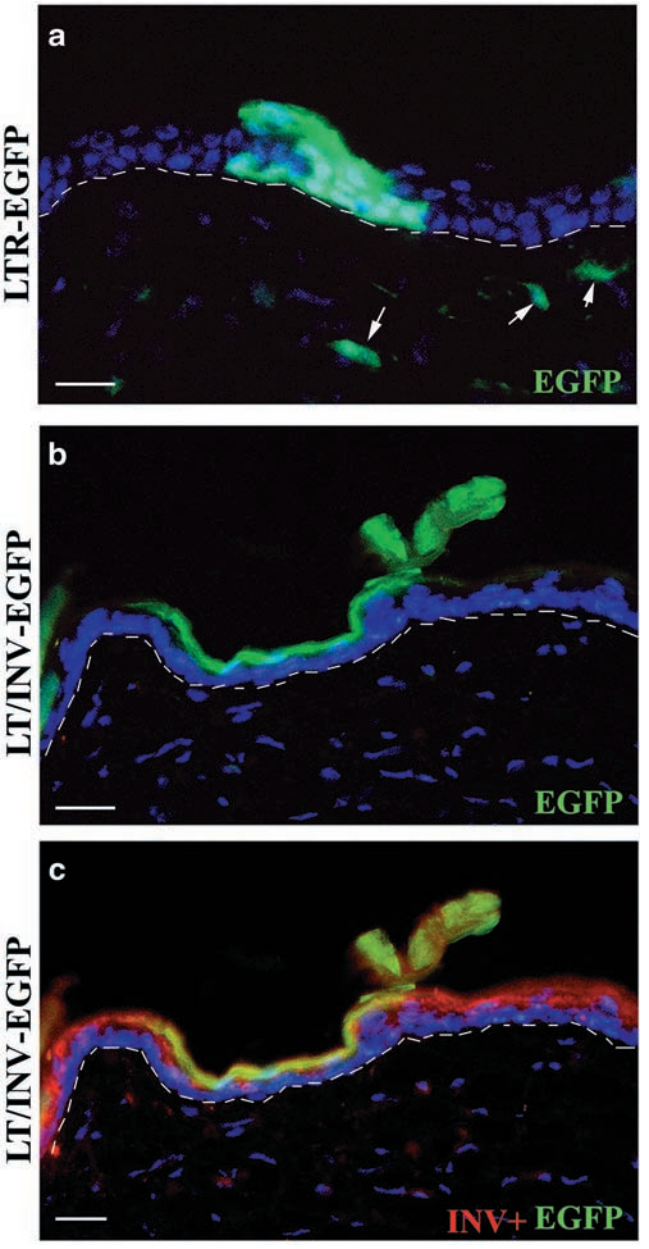


***Figure 5*** *In vivo transduction of mouse dorsal skin with INV/LTR-EGFP retroviral vector. The dorsal skin of immunodeficient SCID mice at 7 weeks of age was depilated and dermabraded. On day 3 after abrasion, 20 μl (containing 2 × $10^7$ viruses) of concentrated LTR-EGFP (a) or INV/LTR-EGFP (b, c) viruses were injected into the hyperproliferative mouse skin. Five months later skin biopsies were taken, fixed in paraformaldehyde and cryosectioned. The sections were analyzed for EGFP expression (green) using fluorescent microscopy. Some sections were immunostained for mouse involucrin (red) to demonstrate the colocalization of endogenous involucrin and retrovirus directed EGFP. All of the sections were counterstained with DAPI (blue). The arrows indicate the EGFP+ fibroblasts and the position of basement membrane is indicated with a dotted line. The bars represent 50 μm.*

the suprabasal layers of epidermis would be lost due to the desquamation of terminally differentiated cells.[6]

Transcriptional control of integrated retroviral vectors is directed by the 5′ LTR of the provirus, which exerts a strong influence on the activity of the involucrin promoter placed within the viral transcription unit.[24] Disabling the LTR by enhancer deletion could partially overcome this transcriptional interference, however strict cell type-specific expression was not observed when the involucrin promoter was placed within the viral transcription of vectors with disabled LTR (unpublished results). In this study, we have tried to redirect rather than disable the 5′ LTR promoter by replacing the constitutive U3 enhancer with a 510-bp enhancer element from the human involucrin gene upper regulatory sequences. Retroviral vectors in which the enhancer-replaced LTR drives expression of a reporter gene (EGFP) were used to transduce human cultured keratinocytes and mouse

epidermal stem cells *in vivo*. Analysis of transgene expression *in vitro* and *in vivo* indicated that the 510-bp involucrin enhancer element effectively restricts expression to the epidermal keratinocytes in a tissue- and stratum-specific manner. In mouse interfollicular epidermis, a stem cell and its descendant transit amplifying cell and the subsequent terminally differentiated cells are organized in a spatially distinct unit, called epidermal proliferative units.[20,21] In the majority of transduced epidermal proliferating units, transcription of the modified LTR appeared to be activated in parallel to the endogenous involucrin promoter. However, in some instances the activity of the hybrid promoter was not tightly regulated. This is likely due to random integration of the retroviral vectors into the genome.[25] Expression of genes introduced by integrating vectors may be affected by local chromatin structure and regulatory elements.[23] The design and incorporation of chromatin insulator sequences into the viral vectors may lead to an improved and more predictable expression of the transgene.

Replacing the viral enhancer appears to be an efficient strategy to restrict transcriptional activity of retroviral vectors to epidermal keratinocytes, although its general applicability needs to be tested with other promoters. The LTR architecture imposes a number of constraints upon the elements that it can accommodate, such as the distance between the enhancer and the promoter and the sequences of the enhancer itself, and it is likely that some tissue-specific promoters will not function properly in this context.[26] The overall size of the inserted promoter is another limitation of this approach. An LTR much larger than its normal size reduces both viral titer and the stability of proviral integration, most likely because of decreased efficiency of reverse transcription.[10] The majority of well-characterized epidermal gene promoters are too large to be incorporated into the LTR and it may be necessary to identify and dissect critical upstream sequences required for keratinocyte and differentiation stage-specific gene expression.[27]

In conclusion, the retroviral vector described in this report may prove valuable in gene transfer applications where transcriptional activation of the gene occurs only after target cell differentiation *in vivo*. The design of similar vectors for various gene therapy applications constitutes an important step towards clinically effective gene therapy.

## Materials and methods

### Transgenic mice

A 754-bp segment of the involucrin promoter was derived from the full-length promoter by deletion of the central *Bgl*II (−2100)/*MSC*I (−192) fragment as depicted in Figure 1. The 754-bp fragment was linked to the β-galactosidase reporter gene and microinjected into C57BL/6 × SJL F2 hybrid mouse eggs (DNX, Princeton, NJ, USA). DNA from pups was screened for the presence of the transgene construct, and from several positive mice a single line was bred with C57BL/6. Tissue samples were harvested from mice and analyzed by histochemical staining for β-galactosidase activity as described previously.[14]

### Plasmid construction

The pINV754-LZ was used as the template to generate the 510-bp fragment of the hINV enhancer by polymerase chain reaction (PCR) using high fidelity Taq polymerase (Roche Molecular Biochemicals, Mannheim, Germany) and the forward primer 5′ AGAATTCTTCTCCA TGTGCATGGG 3′ (−2463 to −2444 relative to the full-length promoter) and the reverse complementary primer 5′ ACTCGAGCTCCAGGTTGAAGGTGATGG 3′(−72 to −52). The restriction sites for *Bam*HI and *Xho*I (underlined) were incorporated to facilitate cloning into the retroviral vector construct. PCR conditions were as follow: 95°C for 3 min; 30 cycles of 95°C for 45 s, 60°C for 45 s, 72°C for 1 min; 72°C for 10 min. The PCR products were purified using the QIAquick PCR purification kit (Qiagen, Hilden, Germany).

LZRS-based vectors have been previously described,[28] and contain Epstein–Barr virus EBNA-1 gene and EBV *oriP cis* elements that allow stable maintenance of retroviral constructs as DNA episomes within retroviral packaging cells. The LZRS-EGFP vector was a gift from Dr Paul Khavari (Stanford University, CA, USA). A shuttle vector containing a single copy of the 3′LTR was constructed from LZRS vector using pBluescript KS. The viral enhancer from the 3′LTR was removed using *Nhe*I and *Xba*I restriction sites to make an enhancer-deleted LTR, and a short DNA fragment containing multiple restriction sites was ligated to facilitate cloning of hybrid enhancers. For construction of the hybrid INV/LTR vector, the viral enhancer from the 3′LTR was replaced with a 510-bp hINV enhancer element. The modified 3′LTR was engineered back to the parental LZRS-EGFP replacing the wild-type 3′LTR as outlined in Figure 2a.

### Recombinant retrovirus production and transduction

293GP retroviral packaging cells were transfected with various vector constructs using the calcium phosphate co-precipitation protocol.[18,29] At 48 h after transfection, cells were passaged into puromycin (1 μg/ml)-containing medium and maintained in the selective medium until 48 h before virus collection. Puromycin-resistant pools of producer cells were transiently transfected with pHCMV-G DNA encoding vesicular somatitis virus-glycoprotein G (gift from Theodore Friedman, UC San Diego, CA, USA). The virus-containing supernatant was collected 36–72 h after transfection, filtered through 0.45-μm pore-size filter (Gelman science, Ann Arbor, MI, USA) and stored at −70°C. For *in vivo* transduction, viral supernatant was filtered and concentrated 1000-fold by ultracentrifugation as described elsewhere.[18] Viral titers were determined on 3T3 or keratinocytes by fluorescence-activated cell sorter (FACS) analysis on a Becton Dickinson FACScan and were between 0.5 and $2.0 \times 10^6$ TU/ml for all viral stocks used for *in vitro* transduction. For *in vitro* transduction, cells were seeded at $3.5 \times 10^4$ cells/well in six-well plates. The next day, recombinant viruses were added at a multiplicity of infection of ~0.8–1 in the presence of 8 μg/ml polybrene (Sigma, St Louis, MO, USA) at 37°C. After 2–3 h, fresh medium was added and 2 days after transduction, cells were either passed or harvested for FACS analysis.

### RT-PCR analysis of viral vectors

Viral RNA was extracted from various viral stocks using QIAamp viral RNA minikit (Qiagen, Hilden, Germany)

using the manufacturer's protocol. For transduced cells, total RNA was isolated using Trizol reagent (Gibco/BRL, Grand Island, NY, USA). First-strand cDNA was synthesized by using MMLV reverse transcriptase (Gibco/BRL) and random primers (N6) in a final volume of 25 μl. Two μl of this reaction was amplified by 30 cycles of PCR (94°C for 3 min; 94°C for 45 s, 60°C for 45 s and 72°C for 45 s; and 72°C for 10 min) using the forward primer 5′CCACCTGTAGGTTTGGCAA GC 3′ and the reverse complementary primer 5′CAGTCAATCGGAGGAC TGGCG 3′ flanking the U3 region of the LTR. To verify the DNA sequences generated by PCR, the fragments yielded were purified and analyzed by restriction mapping.

### *Cell cultures and construction of organotypic cultures*

Human foreskin or adult skin keratinocytes (passage 2–6) were grown in submerged cultures in the presence of irradiated 3T3 cells,[30] using keratinocyte medium described by Wu *et al*[31] supplemented with 5% fetal bovine serum. 293GP,[18] HepG2 (hepatocellular carcinoma; HB-8065), C2C12 (mouse myoblasts; ATCC CRL 1772) and SW480 (colon carcinoma; ATCC CCL-228) were grown in Dulbecco's minimal essential medium (DMEM; Gibco/BRL) supplemented with 10% fetal bovine serum, penicillin (100 U/ml) and streptomycin (100 μg/ml). NIH-3T3 mouse fibroblasts were grown in 10% bovine calf serum in DMEM.

For construction of the organotypic cultures, type I rat-tail collagen at 2 mg/ml (Upstate Biotechnology, Lake Placid, NY, USA) was neutralized on ice by mixing with 10× DMEM and 1 mM NaOH. Human dermal fibroblasts were added to the collagen mixture at $7 \times 10^4$ cells/ml, and 3 ml of the collagen mixture was added to 0.02 μm anopore membrane inserts (Nunc, Naperville, IL, USA) in a six-well culture dish. After 1 h at 37°C, when the gel had formed, the cells were covered with keratinocyte media. The gels were allowed to contract for 1 week. At this time, the media was removed and the transduced keratinocytes were seeded on to the collagen matrix ($2 \times 10^5$ cells/matrix). Keratinocytes were allowed to attach to the matrix for 2 h at 37°C incubator before being submerged in the media and maintained for 4 days before raising to the air–liquid interface for an additional 8 days. Cultures were harvested, washed in PBS, fixed in 4% paraformaldehyde for 30 min on ice, embedded in OCT media and cryopreserved.

### *In vivo transduction*

The dorsal skin of 7-week-old severe combined immunodeficient (SCID) mice (Taconic Farm, NY, USA) was depilated and dermabraded as described previously.[20] On day 3 after abrasion, 20-μl aliquots of concentrated viruses (containing $2 \times 10^7$ transducing units) were injected into the hyperproliferative skin. Initial EGFP expression in the skin was assessed by tape stripping the cornified cells and examining the tapes under fluorescent microscopy. At 22 weeks after transduction skin biopsies were obtained, fixed for 30 min in 4% paraformaldehyde on ice, washed in PBS, soaked for 30 min in 30% sucrose and cryopreserved in OCT-embedding media. Animal studies were performed in accordance with institutional guidelines set out by the State University of New York.

### *Detection of EGFP and involucrin*

Transduced cells grown in submerged cultures were trypsinized, fixed in 2% paraformaldehyde and analyzed for EGFP expression by FACS on a Becton Dickinson FACScan. For detection of EGFP and involucrin in tissue sections, cryosections were fixed in 4% paraformaldehyde for 10 min, blocked with 5% non-fat milk and incubated with either a mouse monoclonal antibody to human involucrin (clone SY5, Sigma) or rabbit polyclonal antibody to mouse involucrin (Covance, Richmond, CA, USA). After extensive washes, sections were treated with biotin-labeled anti-mouse or anti-rabbit antibodies followed by Alexa 594-conjugated avidin (Molecular Probes, Eugene, OR, USA). Slides were mounted with Vectashield mounting media containing DAPI (Vector Laboratories, Burlingame, CA, USA) and were examined via fluorescent microscopy using the proper filters.

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