Retrovirus-Mediated Transduction of Cultured Epidermal Keratinocytes

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Retrovirus-mediated gene transfer is an efficient means of introducing and expressing exogenous gene(s) in many cell types including keratinocytes. However, parameters of transduction and gene expression have not been systematically analyzed for keratinocytes. To carry out such a study we have transduced cultures of newborn foreskin cells with retroviral vectors that encode the genes for neomycin resistance (neo^r) and for beta-galactosidase (B-gal). The neo^r gene is a dominant selectable marker and the B-gal gene encodes a histochemically detectable product. Our key findings are the following: 1) all keratinocytes that form colonies can be successfully transduced at a viral titer greater than 5×10^6 colony-forming units/ml; 2) transduction is effected by integration of a single copy of retroviral DNA; 3) transduced cells are not at a growth disadvantage and, in fact, single clones of transduced keratinocytes can be expanded to yield over 10^9 cells, suggesting that stem cells are transduced; 4) whereas most transduced colonies exhibit B-gal staining in a high percentage of constituent cells, some colonies had a mosaic or sectored staining pattern; 5) expression of the non-selectable B-gal gene was somewhat greater in differentiated cells of the culture as compared to nondifferentiated precursors. The ability to transduce stem cells at a high efficiency and to follow expression of transduced genes in clonal progeny will allow lineage mapping in stratified epithelial tissues. *J Invest Dermatol 97:824–829, 1991*

The expression of exogenous genes in somatic cells has become a powerful tool to study gene regulation as well as a possible approach for somatic cell gene therapy. Recombinant retroviral vectors have become a popular method for introducing such genes primarily because the frequency of gene transfer is usually high. Such vectors have been used to transfer DNA into a number of cell types including fibroblasts [1], bone marrow stem cells [2,3], endothelium [4], and hepatocytes [5]. Keratinocytes have also been targets for retroviral transduction. A retroviral vector containing sequences coding for transforming growth factor-alpha has been transduced and expressed in a mouse keratinocyte cell line [6]. Cultured primary keratinocytes have been transduced with retroviral vectors containing the human growth hormone gene [7], the adenovirus early region 1A gene [8], and the neo^r gene [9]. However, the parameters of retroviral-mediated transduction and gene expression in keratinocytes have not been systematically examined.

The present study was undertaken to characterize frequency of gene transfer, state of viral DNA in the recipient cell, effect of retroviral gene expression on cell viability, and expression of transduced genes during keratinocyte differentiation.

MATERIALS AND METHODS

Cell Culture Human epidermal keratinocytes were cultured from newborn foreskin by the method of Rheinwald and Green [10] in keratinocyte medium described by Wu et al [11]. Cultures were established through trypsinization of foreskin fragments. 3T3 cells and packaging cell lines (to be described) were maintained in Dulbecco's modified Eagle's medium containing 10% calf serum. For some experiments, neo^r-3T3 cells were used for substrate support. A stable line of neo^r-3T3 was generated by transfecting 3T3 with pSV2NEO DNA and selecting with G418.

Retroviral Vectors and Producer Lines Retroviral vectors LJ [12] and LXSN [13] contain the neomycin phosphotransferase gene that confers neomycin resistance (neo^r). The BAG [14] vector contains the neo^r gene as well as the bacterial gene for beta-galactosidase (B-gal) (Fig 1). All retroviral vectors are derived from the Moloney murine leukemia virus. Infectious recombinant retrovirus was generated in two steps: 1) by transfecting the recombinant plasmid DNA into the ecotropic packaging cell line psi-cre; and 2) by using the supernatant from these cells to infect the amphotropic packaging cell line PA-317 (LXSN), ψ-AM (BAG), or ψ-crip (LJ) [13]. Infection was performed with fresh, filtered (0.45 μ, Gellman) supernatant from confluent amphotropic lines. Assays for viral titers and for amphotropic helper virus were performed as previously described [15]. All amphotropic packaging cell lines were helper-

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Abbreviations:

B-gal: beta galactosidase
BSA: bovine serum albumin
CAT: chloramphenicol acetyltransferase
CFE: colony-forming efficiency
G418: G-418 sulfate antibiotic (Geneticin)
LTR: long terminal repeat of Moloney murine leukemia virus
neo^r: neomycin resistance
NP40: Nonidet 40
NS: nonselected transduced keratinocytes
ONPG: o-nitrophenyl-B-D-galactopyranoside
PBS: phosphate-buffered saline
SEL: G418-selected transduced keratinocytes
X-Gal: 5-bromo-4-chloro-3-indolyl B-D-galactoside

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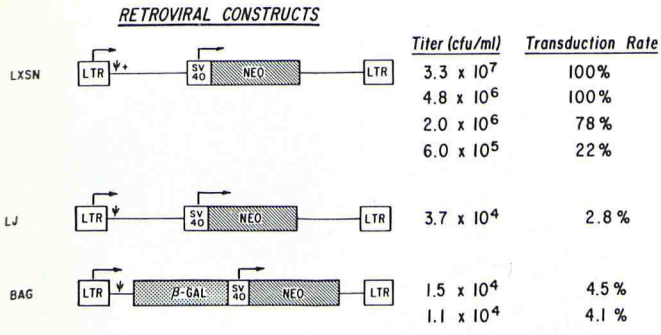

Figure 1. Structure, titers, and keratinocyte transduction rates of retroviral vectors. Retroviral vector titers were determined by infecting 3T3 cells with filtered supernatant from confluent amphotropic packaging lines and counting the number of G418-resistant colonies transduced per milliliter of viral supernatant (cfu/ml). Transduction rates were determined by infecting keratinocytes 24 h after plating. LTR, long terminal repeat of Moloney murine leukemia virus; ψ, packaging signal; ψ^+, extended packaging signal; Γ, transcription initiation sites.

virus free. Efforts to augment titers by centrifugation or lyophylizations were unsuccessful.

Transduction of Human Keratinocytes and Determination of Keratinocyte Transduction Rate The rate at which keratinocytes were transduced was determined by plating keratinocytes at low density onto a neo^r-3T3 feeder layer in 6-cm dishes. Keratinocytes were seeded at densities of 6×10^2 to 6×10^4 per dish and infected 24 h later with 2 ml of viral supernatant that contained Polybrene (8 μg/ml, Sigma) for 2 h. The volume was then brought to 10 ml by the addition of fresh media. Three to four days later, the cultures were fed media for 7–10 d containing the aminoglycoside antibiotic G-418 sulfate (G418, 0.8 mg/ml active, Gibco) that is toxic to cells that do not have the neo^r phenotype. Plates were then stained with crystal violet and colonies were counted. Transduction rate was calculated as follows:

$$\text{Transduction rate} = \frac{\text{number of neo}^r \text{ colonies}}{(\text{number of cells plated})\ (\text{CFE})}.$$

For all other experiments, keratinocytes were infected using virus-producing packaging cells as feeder cells. Packaging cells (4×10^6 per 10 cm dish) were inactivated with Mitomycin-C (5 μg/ml for 2 h), a procedure that did not significantly reduce virus output. Selection for neo^r keratinocytes was performed by adding G418 (0.8 mg/ml active) to the culture 3 to 4 d after seeding.

Clonal Expansion of Transduced Cells and Southern Blot Analysis Two weeks after infection with LJ and selection with G418, individual clones were isolated using cloning cylinders and expanded. Total cellular DNA was isolated from expanded clones [16] was treated with Bam H1, electrophoresed, and probed with P^{32}-labeled neo^r DNA [16]. This combination of restriction and probe resulted in a single band for each integrated provirus. The Southern hybridization procedure has been previously described [17].

X-Gal Staining of Cultured Keratinocytes B-galactosidase expression was detected by staining with 5-bromo-4-chloro-3-indolyl B-D-galactopyranoside (X-Gal, Boeringer Mannheim) according to the protocol outlined by Cepko [15]. Following two rinses in PBS, cells were fixed for 15 min in 1% glutaraldehyde. X-Gal buffer was prepared containing 0.01 M Na_2HPO_4 buffer, 0.15 M NaCl, 35 mM $K_3Fe(CN)_6$, 35 mM $K_4Fe(CN)_6 \cdot 3H_2O$, 1 mM $MgCl_2$, and 1 mg/ml X-Gal dissolved in dimethyl formamide. Following filtration through a 0.45 μ filter to remove particulate material, cultures were incubated with X-Gal buffer for 0.5 to 2 h. Non-transduced keratinocytes were stained to assess endogenous B-Gal activity.

Ficoll Gradient Separation and Analysis of B-Galactosidase Staining of Fractions Transduced keratinocyte cultures selected with G418 (SEL) or not subject to G418-selection (NS) were fractionated in Ficoll 400 (Pharmacia) gradients prepared in 50 ml conical plastic centrifuge tubes (Falcon) by successive layering of 10 ml aliquots of 20%, 15%, 10%, and 5% Ficoll in PBS containing 0.5 mg/ml BSA. Both NS and SEL keratinocyte cultures were disaggregated by treatment in 0.02% trypsin (Sigma) and washed once in media containing serum and once in PBS. A suspension of 10^7 cells in 5 ml PBS containing 10 mg/ml BSA was layered on top of a gradient and centrifuged at 2200 rpm (IEC, model HN-S) for 90 min at 7°C [18]. Distinct bands of cells formed at the interfaces between layers and these were recovered and suspended with 40 ml PBS. The cells of each fraction (large, intermediate and small) were collected by centrifugation and resuspended in PBS. Cell counts were performed in a Coulter counter (model ZB1). To determine the percentage of B-gal positive cells, 2×10^4 cells were resuspended in 1% glutaraldehyde fixative, rinsed in PBS and stained with X-Gal. To determine the average B-gal activity per cell, 10^4 cells were resuspended in PBS containing 3.5 mM ONPG (Sigma) and 0.5% NP-40 for 1 h at room temperature. The samples were centrifuged at 10,000 rpm for 2 min and activity in the supernatant was measured spectrophotometrically (Brinkman) at 420 nm.

RESULTS

Efficiency of Transduction Two types of retroviral vectors were used to transduce keratinocytes, LXSN and LJ. LXSN contains an extended psi-packaging sequence and produced titers that ranged from 6×10^5 to 3.3×10^7 cfu/ml (Fig 1). LXSN-mediated transduction of keratinocytes with viral titers greater than 5×10^6 cfu/ml resulted in all clonogenic cells forming neo^r colonies indicating 100% transduction efficiency (Fig 2). The LJ vector has a shorter psi-packaging sequence and viral titers were considerably lower (3.7×10^4 cfu/ml). However, even at this low titer, 2.8% of the clonogenic keratinocytes were transduced. The addition of the B-gal sequence to LJ lowered the titer to 1.1×10^4. Retroviral titers below 10^4 cfu/ml produce too few transductants to be useful.

Single Hit Kinetics of Transduction To determine the number of copies of proviral DNA in transduced cells, keratinocytes were transduced with LJ at a transduction rate of 2.8% and 10 neo^r colonies were isolated and expanded. DNA from each was digested with Bam H1 and analyzed by Southern hybridization using a labeled probe of neo^r DNA (Fig 3). Bam H1 would cut proviral DNA at one defined site within the vector and at another undefined site in flanking cellular DNA. Thus for each copy of provirus a unique and single band will be produced. The results show that, in each clonal isolate, there was one copy of integrated vector DNA, indicating single hit kinetics. The variation in molecular weight of the labeled

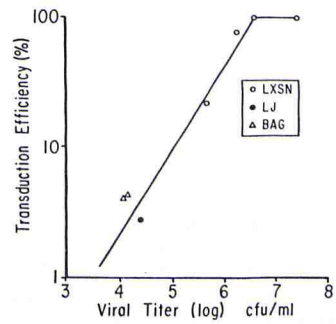

Figure 2. Relation between viral titer and keratinocyte transduction rate. The data in this figure are taken from Table I and plotted on logarithmic axes.

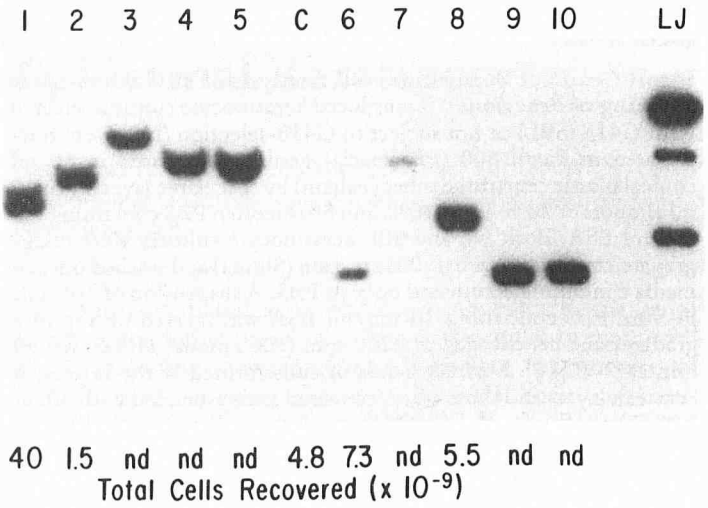

Figure 3. Analysis of proviral integration of retrovirus vector in transduced human keratinocytes. Equal amounts of cellular DNA from LJ-transduced and G418-selected keratinocyte clones were digested with Bam H1 and analyzed by Southern blot with a P^{32}-labeled neo^r gene probe. Restriction with this enzyme produced a unique band for each proviral integration. *Lanes 1–10,* DNA from transduced and selected clones expanded by four to six serial passages. *Lane C,* DNA from a nontransduced clone. *Lane LJ,* control LJ plasmid DNA. For some clones, the total number of cells recovered after four to six passages is noted in the lower portion of the figure. For each of the other clones, the total number of cells recovered was not determined and is labeled nd.

Bam H1 fragments among clonal isolates also suggests that proviral integration was random. The lower signal intensity seen in lanes 6 and 7 may be due to differing degrees of stability of integrated vector in each clone. Since clones were selected initially in G418 yet expanded without selection, vector-deleted revertants may have been generated in these clonal isolates.

B-Gal Expression in Keratinocyte Colonies The BAG vector contains two genes of interest, neo^r and B-gal, and transduced cells can be selected by the neo^r phenotype. To determine if the second, nonselectable B-gal gene was co-expressed, keratinocytes were transduced with BAG and stained for B-gal expression after G418 selection. Expression of the B-gal gene was detected by a cytoplasmic blue-green color. Most selected neo^r colonies were completely stained for B-gal indicating co-expression of the nonselectable gene. In transduced cultures that were not G418-selected, completely stained colonies were observed (Fig 4*A*), and in addition, some colonies were completely unstained (Fig 4*A*). These unstained colonies were likely to be nontransduced cells. A small number of colonies had a mosaic staining pattern where only a portion of the cells stained for B-gal (Fig 4*B*).

When transduced cultures were passaged, colonies with a sectored staining pattern were observed in which a cluster of contiguous cells stained positively (Fig 4*C*). These sectored colonies

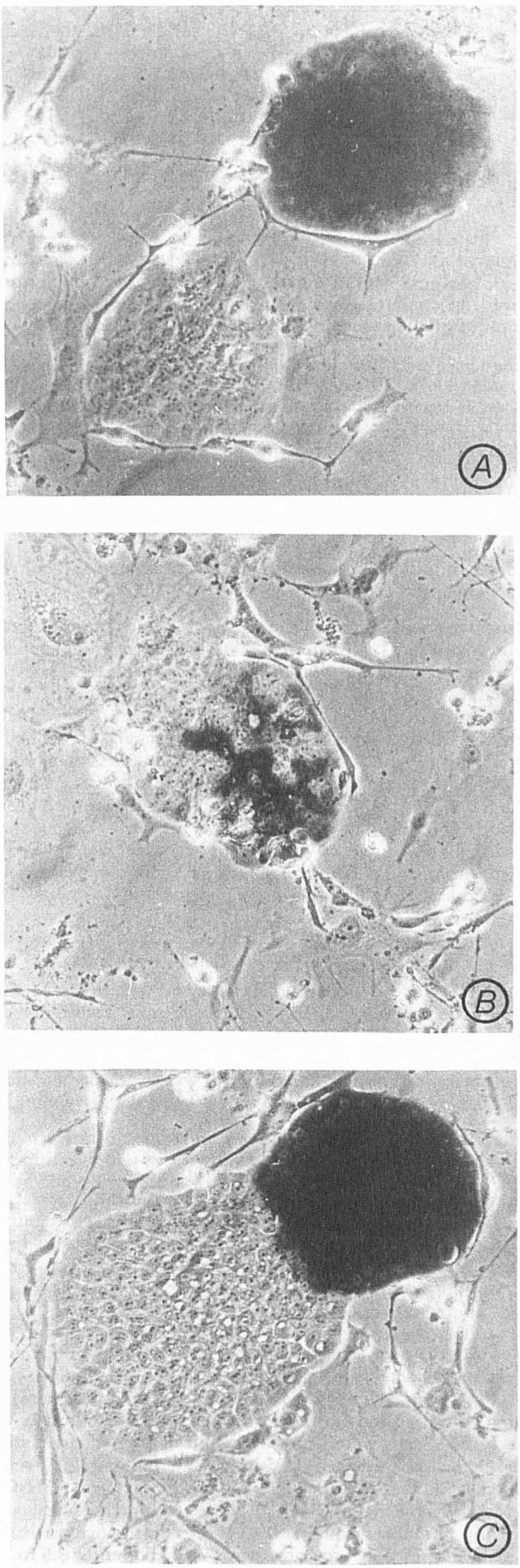

Figure 4. Pattern of B-gal expression in keratinocytes. Cultured keratinocytes were transduced with BAG vector by cocultivation with packaging cells, grown to confluence and passaged to a Mitomycin C-treated feeder layer. After 4 to 8 cell doublings, cultures were stained for B-gal activity as described. The characteristic patterns of B-gal expression are as follows. *A)* In one colony, all cells stained positive for B-gal activity and in a nearby colony, no cells stained positive for B-gal. *B)* Colony demonstrating a mosaic pattern of expression. *C)* Colony demonstrating a sectored staining pattern. (Magnification ×250.)

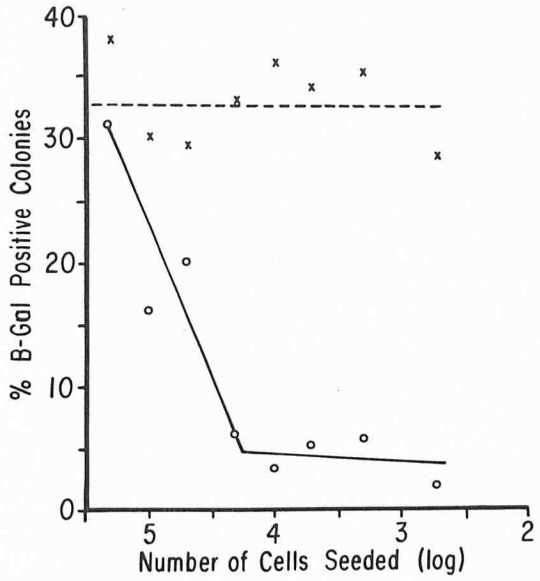

Figure 5. Percentage of B-gal positive colonies as a function of the number of cells seeded. A culture of nonselected (NS), BAG-transduced keratinocytes containing a mixture of B-gal positive and B-gal negative cells was disaggregated and varying numbers were plated onto Mitomycin C-treated 3T3 feeder layers. After 4 to 6 cells doublings, cultures were fixed and stained for B-gal. The percentage of B-gal positive colonies with a sectored appearance (O) and those entirely B-gal positive (X) are plotted.

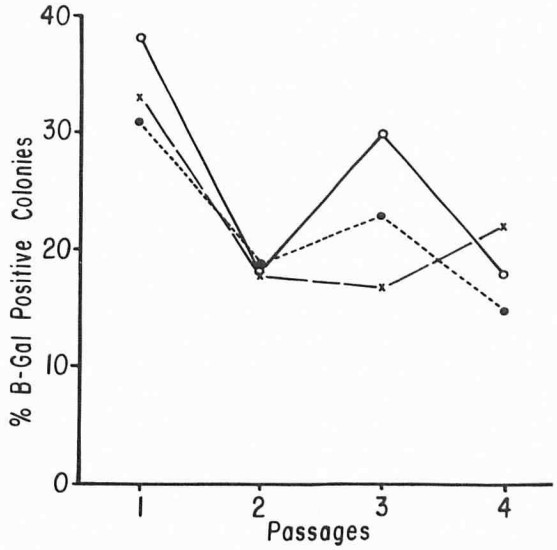

Figure 6. Retroviral transduction of cultured keratinocytes does not result in a selective growth disadvantage to transduced cells. Keratinocytes were transduced with BAG by cocultivation with Mitomycin C-treated BAG packaging cells for 5 d. No G418 selection was applied. The keratinocytes were further passaged 4 times using regular 3T3 feeder cells. At each passage, a culture was stained with X-Gal and the percentage of B-gal positive colonies measured. The results of three experiments were plotted.

persisted through consecutive passaging under non-selective conditions and appeared to have arisen as a result of aggregation of transduced and nontransduced clonogenic cells. This was confirmed by the decrease in sectored colonies as the total number of cells seeded was also decreased (Fig 5). There was no change in the percentage of fully B-gal positive colonies indicating that dilution itself did not select against B-gal–expressing clonogenic cells.

Growth Capacity of Transduced Keratinocytes To determine if transduced keratinocytes were at a selective growth disadvantage, keratinocytes transduced with BAG were maintained through four passages without any selection for neo^r. At each pass the percentage of B-gal positive colonies was noted. As seen in Fig 6, the percentage of keratinocyte colonies expressing B-gal remained relatively constant throughout the four passages. This result suggests that transduced keratinocytes were not at any growth disadvantage nor were they selected against with cell passage. The constant proportion of B-gal positive colonies also indicated that B-gal expression was stably inherited by successive progeny.

To determine the growth potential of transduced keratinocytes, five clones of LJ-transduced keratinocytes examined in Fig 3 were expanded by serial passage. The number of cells harvested after four to six passages is listed in the lower portion of Fig 3. A nontransduced clone produced 4.8×10^9, whereas four transduced clones produced 1.5×10^9 to 4.0×10^{10} cells.

Expression of B-Gal During Keratinocyte Differentiation Cultured human keratinocytes undergo differentiation. To gauge if expression of B-gal in transduced keratinocytes changes during differentiation, the cells in transduced cultures were fractionated in gradients of Ficoll 400 and B-gal activity assayed. Sedimentation in Ficoll 400 separates the cells into three fractions: small undifferentiated cells, large differentiated cells, and an intermediate fraction. Two measurements of B-gal activity were made on cells from each fraction: the percentage of B-gal positive cells to assess what fraction of cells expressed the transduced gene and, the total B-gal activity in 10^4 cells to provide a measure of the average amount of B-gal activity per cell (Table I). Because differences were noted in the pattern of B-gal positive cells when transduced cultures were selected with G418, analysis of B-gal expression during differentiation was performed with nonselected (NS) and selected (SEL) cultures. As expected, the percentage of B-gal positive cells in SEL cultures was two- to threefold higher than in NS cultures (Table I). In both NS and SEL cultures, keratinocytes in all three fractions expressed B-gal. For NS keratinocytes, a two- to fourfold increase in average B-gal activity per cell was seen in the large cell fraction when compared to the small cell fraction, even though all three fractions showed similar percentages of B-gal positive cells. For SEL cultures, a small increase in average B-gal activity was noted in the large cells but the significance of this is questionable as this increase was noted in only one of the two trials. Thus, differentiated transduced kerati-

Table I. B-gal Activity in Transduced Keratinocytes at Various Stages of Differentiation[a]

Fraction	G418-selected		Nonselected	
	Trial I	Trial II	Trial I	Trial II
	Percent B-gal positive cells			
Small	84	74	25	22
Intermediate	70	77	29	23
Large	60	62	28	20
	Average B-gal activity/cell			
Small	2.6	11.0	0.9	6.0
Intermediate	3.5	10.6	1.3	11.0
Large	3.7	9.1	3.4	10.0

[a] B-gal activity in transduced keratinocytes at various stages of differentiation. Nonselected (NS) and selected (SEL), BAG-transduced keratinocytes were grown to confluence, trypsinized, and fractionated in gradients of Ficoll 400 as described. Small cells are basal cells. Large cells are differentiated suprabasal cells. The intermediate cells contain a mixture of the two types. Fractionated cells were fixed and stained for B-gal to determine the percentage of B-gal positive cells in each fraction. The average B-gal activity per cell was assessed by incubating 10^4 cells from each fraction in 3.5 mM ONPG with 0.5% NP40 for 1 h and measuring the absorbancy at 420 nm. Absorbance readings have been multiplied by 100. Two independent trials were performed for all analyses.

nocytes contained higher amounts of B-gal activity than their precursor nondifferentiated cells.

DISCUSSION

Retroviruses, like all other viruses, have evolved mechanisms for efficient transfer and expression of their genetic material in the host cell. Retroviruses are RNA viruses whose genomes are reverse transcribed and integrated as proviral DNA in the host chromosomal DNA [19]. In the integrated state, RNA transcribed from the provirus directs translation of viral products and is encapsidated to form viral progeny. To utilize retroviruses as a vector, genes essential for viral replication have been deleted and replaced by a gene or genes of interest. Infectious but replication-defective retrovirus is produced by transfecting recombinant viral DNA into "packaging" cell lines that have been engineered to express the missing viral genes necessary for packaging but not replication [20]. The advantages of retroviral vectors are their high efficiency of gene transfer, their wide host range, their precise integration, and their lack of vector spread after transduction.

Although retroviruses have been used for gene transfer with a variety of cell types, their use with keratinocytes is complicated by the fact that successful infection occurs only in replicating cells [21]. Replicating keratinocytes in the epidermal proliferation unit of intact epidermis comprise two populations, stem cells that cycle slowly and transient amplifying cells that replicate more frequently and give rise to terminally differentiated cells [22]. In confluent, steady-state cultures of keratinocytes, a similar functional organization is present. There are slowly and rapidly cycling cells [23] and the rapidly cycling cells are thought to undergo terminal differentiation [24]. Targeting transduction to slowly cycling stem cells may therefore be difficult. To overcome this potential problem, transduction was performed on keratinocytes 24 h after seeding. Previous kinetic studies show that at this point in time, cells are doubling every 16 h with no detectable cell-cycle heterogeneity and no terminal differentiation [23,25]. Indeed, when the titers of retrovirus vector were 5×10^6 or higher, all clonogenic cells were transduced, i.e., transduction efficiency was 100% (Figs 1 and 2). Furthermore, transduced cells were at no growth disadvantage (Fig 6) and expansion of four transduced colonies by serial passage resulted in yields ranging from 1.5×10^9 to 4×10^{10}. It would appear that stem cells have indeed been transduced in culture. This conclusion could be further supported by demonstrating the phenotype of transduced cells as holoclones as defined by Barrandon and Green [26]. Holoclones have a characteristic high colony-forming efficiency and extended growth potential in culture. It is unlikely that the extended growth capacity seen in the transduced cells of our experiments resulted from immortalization of cells of limited growth potential secondary to retroviral transduction. Barrandon et al [8] have shown that paraclones, clonogenic cells with a short replicative life span, are not altered by retroviral transduction unless these vectors contain a transforming gene such as the adenovirus early region 1A. In addition, retrovirus-mediated transduction does not result in a selective disadvantage to these cells in culture as seen by the fact that the percentage of B-gal positive colonies changed little over four passages (Fig 6). In vivo, canine keratinocytes transduced with a neo^r-containing retrovirus vector persist in the transplanted canine skin for 130 d and may therefore not be at a selective disadvantage either [9].

The BAG retroviral vector used in this study was engineered to transfer the gene for neo^r as well as the gene for B-gal. The B-gal gene expresses a histochemically detectable product that is a non-selectable marker. Acquisition of a neo^r phenotype on the other hand, allows selection of transduced cells from amongst a background of nontransduced cells. This might be very useful in situations in which transduction of keratinocytes is expected to be infrequent, as described above. Colonies of keratinocytes selected by their neo^r phenotype generally exhibit B-gal staining in over 95% of the cells. However, a small but significant population of neo^r colonies expressed B-gal in a mosaic pattern as seen in BAG-transduced NIH 3T3 fibroblasts [14]. A mosaic pattern could arise by transduction of some of the cells in a multicell colony. A mosaic pattern could also arise as a result of failure of the nonselectable gene to be expressed. The neo^r gene in the BAG retroviral vector is regulated by an internal SV40 promoter, whereas the B-gal gene is regulated by the upstream retroviral long terminal repeat (LTR). Gene expression from such an upstream promoter in integrated retrovirus vectors has been shown to be suppressed when expression from the internal gene is selected for [27]. This suppression is thought to be epigenetic [28]. Finally, B-gal in some cells may be below the level of histochemical detection. Fluorescence-activated cell sorting, for example, can detect levels of B-gal activity that cannot be detected by X-Gal cytochemical stain [29].

In cultures that had been transduced with the BAG vector and passaged without G418, "sectored" colonies were seen (Fig 4C). The appearance of these colonies suggested that they have resulted from aggregation of B-gal positive and B-gal negative clonogenic cells. In support of this, we found that the percentage of sectored colonies diminished with increasing cell dilution at the time of plating. Sectored colonies were present when as few as 500 keratinocytes were plated on a 6-cm dish. This suggests that the only method to ensure clonal growth is by seeding a single keratinocyte on a dish, as was done by Barrandon and Green [25].

The clonal progeny of vector-transduced keratinocytes can be identified by the unique integration pattern of proviral DNA following restriction and Southern blotting of DNA. The sizes of the viral DNA-containing fragments vary with the distances between the Bam H1 site in the integrated vector and the closest Bam H1 site in flanking genomic DNA. Our results suggest that viral integration in keratinocytes is random in nature because clonal progeny of LJ-transduced keratinocytes each demonstrate different integration patterns. No sequence-specific integration sites could be identified as have been noted in fibroblasts [30]; however, a considerably larger number of integration sites would need to be studied in keratinocytes to rule out this possibility. This does not exclude the possibility that the vector integrates within specific regions of the cellular genome. Proviral integration sites may be used as a clonal marker in future studies of the lineage of retrovirally infected cells.

To determine if expression of the B-gal gene was altered during keratinocyte differentiation, cultures transduced with BAG were fractionated in gradients of Ficoll 400. The percentage of cells expressing B-gal activity was generally the same for the small, intermediate and large cell fractions (Table I); however, in nonselected cultures the average B-gal activity, as measured by B-gal activity in aliquots of large or small cells containing the same number of cells, was greater in the large cell fraction. The simplest explanation for this increased B-gal activity is accumulation during terminal cell differentiation. Other factors may, however, be operating. In an earlier study, transient expression of a reporter gene, CAT, was also higher in the large cell fraction of keratinocyte cultures when this construct was introduced by transfection [18]. In this previous study, the CAT gene was regulated by the LTR of Rous sarcoma virus. Several possible explanations, including accumulation of CAT protein in large cells, were considered and rejected for various reasons. It appeared that the increased CAT activity was the result of an increased level of gene expression in the large cell fraction. Subsequently, we found that CAT mRNA levels were indeed highest in the large cell fraction [31]. The higher levels of B-gal activity seen in the current study in the transduced large cells could therefore have arisen from increased levels of B-gal mRNA as well as by accumulation of this protein.

In summary, the strategy for retroviral transduction of human keratinocytes in culture has several features that favor its use. These include high efficiency of gene transfer, transduction of stem cells, integration of single provirus, and no apparent deleterious effect on cell growth. Expression of a non-selected gene occurs in both differentiated and nondifferentiated cells. However, expression is somewhat higher in the differentiated population and this may complicate studies in which promoters known to be expressed specifically in differentiating keratinocytes in vivo are tested by retroviral transduction in culture. The ability to genetically mark stem

cells will also allow lineage mapping of their clonal progeny in stratified epithelial tissues.

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REFERENCES

1. St. Louis D, Verma IM: An alternative approach to somatic cell gene therapy. Proc Natl Acad Sci USA 84:3150–3154, 1988
2. Bender MA, Gelinas RE, Miller AD: A majority of mice show long-term expression of a human B-globin gene after retrovirus transfer into hematopoietic cells. Mol Cell Biol 9:1426–1434, 1989
3. Stead RB, Kwok WW, Storb R, Miller AD: Canine model for gene therapy: inefficient gene expression in dogs reconstituted with autologous marrow infected with retroviral vectors. Blood 71:742–747, 1988
4. Nabel EG, Plautz G, Nabel GJ: Site-specific gene expression in vivo by direct gene transfer into the arterial wall. Science 249:1285–1288, 1990
5. Wilson JM, Jefferson DM, Chowdhury JR, Novikoff PM, Johnston DE, Mulligan RC: Retrovirus-mediated transduction of adult hepatocytes. Proc Natl Acad Sci USA 85:3014–3018, 1988
6. Finzi E, Fleming T, Pierce JH: Retroviral expression of transforming growth factor-alpha does not transform fibroblasts or keratinocytes. J Invest Dermatol 95:382–387, 1990
7. Morgan JR, Barrandon Y, Green H, Mulligan RC: Expression of an exogenous growth hormone gene by transplantable human epidermal cells. Science 237:1476–1479, 1987
8. Barrandon Y, Morgan JR, Mulligan RC, Green H: Restoration of growth potential in paraclones of human keratinocytes by a viral oncogene. Proc Natl Acad Sci USA 86:4102–4106, 1989
9. Flowers MED, Stockschlaeder MAR, Schuening FG, Niderwieser D, Hackman R, Miller AD, Storb R: Long-term transplantation of canine keratinocytes made resistant to G418 through retrovirus-mediated gene transfer. Proc Natl Acad Sci USA 87:2349–2353, 1990
10. Rheinwald JG, Green H: Serial cultivation of strains of human epidermal keratinocytes: the formation of keratinizing colonies from single cells. Cell 6:331–343, 1975
11. Wu Y-J, Parker LM, Binder NE, Beckett MA, Sinand JH, Griffiths CT, Reinwald JG: The mesothelial keratins: a new family of cytoskeletal proteins identified in cultured mesothelial cells and nonkeratinizing epithelia. Cell 31:693–703, 1982
12. Korman AJ, Frantz JD, Strominger JL, Mulligan RC: Expression of human class II major histocompatibility complex antigens using retrovirus vector. Proc Natl Acad Sci USA 84:2150–2154, 1987
13. Miller AD, Rosman GJ: Improved retroviral vectors for gene transfer and expression. Biotechniques 7:980–990, 1989
14. Price J, Turner D, Cepko C: Lineage analysis in the vertebrate nervous system by retrovirus-mediated gene transfer. Proc Natl Acad Sci USA 84:156–160, 1987
15. Cepko C: Lineage analysis and immortalization of neural cells via retrovirus vectors. In: Boulton AA, Baker GB, Campagnoni AT (eds.). Neuromethods, Vol. 16. Molecular and Neurobiological Techniques. The Humana Press, Clifton, New Jersey, 1989, pp 177–219
16. Sambrook J, Fritsch EF, Maniatis T: Molecular Cloning: A Laboratory Manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1989, pp 9.36–9.62
17. Southern EM: Detection of specific sequences among DNA fragments separated by gel electrophoresis. J Mol Biol 98:508–517, 1975
18. Lee JI, Taichman LB: Transient expression of a transfected gene in cultured epidermal keratinocytes: implications for future studies. J Invest Dermatol 92:267–271, 1989
19. Varmus H: Retroviruses. Science 240:1427–1435, 1988
20. Miller AD: Retrovirus packaging cells. Human Gene Therapy 1:5–14, 1990
21. Miller DG, Adam MA, Miller AD: Gene transfer by retrovirus vectors occurs only in cells that are actively replicating at the time of infection. Mol Cell Biol 10:4239–4242, 1990
22. Potten CJ: Cell replacement in epidermis (keratopoiesis) via discrete units of proliferation. Int Rev Cytol 69:271–318, 1981
23. Albers KM, Setzer RW, Taichman LB: Heterogeneity in the replicating population of cultured human epidermal keratinocytes. Differentiation 31:134–140, 1986
24. Albers KM, Greif F, Setzer RW, Taichman LB: Cell-cycle withdrawal in cultured keratinocytes. Differentiation 34:236–240, 1987
25. Barrandon Y, Green H: Cell size as a determinant of the clone-forming ability of human keratinocytes. Proc Natl Acad Sci USA 82:5390–5394, 1985
26. Barrandon Y, Green H: Three clonal types of keratinocyte with different capacities for multiplication. Proc Natl Acad Sci USA 84:2302–2306, 1987
27. Emerman M, Temin HM: Quantitative analysis of gene suppression in integrated retrovirus vectors. Mol Cell Biol 6:792–800, 1986
28. Emerman M, Temin H. Genes with promoters in retrovirus vectors can be independently suppressed by an epigenetic mechanism. Cell 39:459–467, 1984
29. Nolan GP, Fiering S, Nicolas J-F, Herzenberg LA: Fluorescence-activated cell analysis and sorting of viable mammalian cells based on B-D-galactosidase activity after transduction of Escherichia coli lacZ. Proc Natl Acad Sci USA 85:2603–2607, 1988
30. Shih C-C, Stoye JP, Coffin JM: Highly preferred targets for retrovirus integration. Cell 53:531–537, 1988
31. Fenjves ES, Lee JI, Garlick JA, Gordon DA, Williams DL, Taichman LB: Prospects for epithelial gene therapy. In: Sutherland BM, Woodhead AD (eds.). DNA Damage and Repair in Human Tissues. Plenum Press, New York, 1990, pp 215–223