Susceptibility of Cell Populations to Transduction by Retroviral Vectors†

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Retroviral transduction efficiency is related to the multiplicity of infection and the physiological state of the target cells. It is generally not known what proportion of a cell population is susceptible to transduction. We used coinfection with two retroviral vectors containing the marker genes for green fluorescent protein and the truncated human nerve growth factor receptor. In the CD34⁺ cell line TF-1 or human primary CD34⁺ hematopoietic progenitor cells, it was found that cells transduced with one vector had a better than random chance of transduction by the other vector. A probability model was developed to estimate target cell susceptibility; susceptibility was calculated as the product of the proportions of transgene-positive cells divided by the proportion of double-positive cells. By using this relationship, it was found that susceptibility was related to the target cell type and culture conditions but not the retroviral titer or the retroviral packaging envelope protein used in this study. Cotransduction with two vectors is a relatively simple procedure that provides a means to assess the maximum transduction level possible in a given cell population.

Gene therapy aims to correct genetic defects through the introduction of genetic material into human cells and tissues. Retroviral transduction of hematopoietic progenitor cells (HPC) is a conceptually attractive methodology aimed at introducing gene constructs that will be passed on to progeny cells, thereby achieving persistent expression of a therapeutic gene (2, 17). Stable virion-producing cell lines can be generated by using established techniques, and the supernatant can be harvested to obtain a population of retroviral particles that will stably infect target cells, generally resulting in a low proviral copy number (<3) (12, 13).

The main limitation of oncoretroviruses is that, for efficient transduction, they require cells to transit the cell cycle shortly after exposure to a virus (9, 15, 16). Because HPC are generally quiescent, they require mitogenic stimulation with hematopoietic growth factors for transduction to take place (7). It is not unreasonable to expect that transduction levels are affected not just by the degree of virus-cell contact but also by the proportion of the cell population that is susceptible to retroviral integration and expression.

Efficient retroviral transduction is important for effective gene-based techniques and potential gene therapies. To facilitate the optimization of transduction protocols, it is useful to be able to identify which factors limit transduction efficiency. MacNeill and coworkers examined whether transduction efficiency is limited by interference between coinfecting retroviruses. They found partial inhibition of infection in both human erythroleukemia (HEL) cells and primary human CD34⁺ HPC

when two vectors targeting the amphotropic receptor were used simultaneously. By contrast, no interference was found when vectors targeting the amphotropic receptor and the gibbon ape leukemia virus (GALV) receptor Pit-1 were used concurrently (11).

In the present work, when a target cell population was simultaneously infected with a mixture of two retroviral vectors at a multiplicity of infection at which the likelihood of receptor interference was low, we observed that cells transduced with one vector had a better than random chance of transduction by the other vector. In this paper, we characterize the codependence of transgene expression and describe a method for estimating the proportion of cells in a population that are susceptible to retroviral transduction.

MATERIALS AND METHODS

Retroviral vectors. The green fluorescent protein (GFP)-containing vector LGFP was produced by cloning GFP565T (Clontech) downstream of both the packaging site and the residue of the *gag* gene that remains in the L9XL retroviral vector. L9XL was constructed by deletion of *neo* from LNL6 (12) and insertion of a polylinker with multiple cloning sites. The vector containing nerve growth factor receptor (NGFR), LNGFR, was generously provided by A. Bower (University of New South Wales, Sydney, Australia) and was constructed similarly to LGFP, by the insertion of cDNA encoding the truncated gene for human NGFR into a derivative of the LNL6 vector with *neo* deleted.

Clonal lines of PA317 (13) and PG13 (14) packaging cells were established and used to produce stocks of virus-containing media (VCM) by filtering cell supernatant through a 0.45- μ m-pore-size filter (Millipore, Sydney, Australia). The retroviral stocks that were produced were PG13/LGFP, PG13/LNGFR, PA317/LGFP, and PA317/LNGFR. Retroviral titers were determined at a limiting dilution of VCM tested on NIH 3T3 cells, by using flow cytometry to determine the number of cells that expressed GFP or NGFR (see below), and expressed as the number of infectious retroviral particles (ivp) per milliliter.

TF-1 cell culture and transduction. All cell lines were obtained from the American Type Culture Collection. TF-1 cells (6) were grown in RPMI 1640 medium (Gibco, Invitrogen, Mt. Waverly, Victoria, Australia) supplemented with 10% fetal bovine serum (Invitrogen) and 2 ng of granulocyte-macrophage colony-stimulating factor (R&D Systems, Minneapolis, Minn.) per ml. They were

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harvested during the exponential growth phase and resuspended at 5×10^5 /ml in a mixture of VCM produced by either PA317 or PG13. LGFP and LNGFR VCM derived from either PG13 or PA317 were diluted with TF-1 culture medium and 16 μ g of Polybrene per ml to yield different ratios of the LGFP and LNGFR vectors. These ratios are detailed in Results. The transductions were conducted at 32°C for 4 or 5 h; the cells were harvested, resuspended in TF-1 culture medium, and cultured for an additional 48 h at 37°C; and then GFP and NGFR expression was examined by flow cytometry.

Isolation of primary CD34⁺ cells. Granulocyte colony-stimulating factor-mobilized peripheral blood was obtained from healthy volunteers or patients attending the Hematology Unit of St. Vincent's Hospital, Sydney. Cord blood was obtained from the umbilical vein following vaginal delivery of full-term babies, and informed consent was obtained from all mothers. The Research and Ethics Committees of the South Eastern Sydney Area Health Service and the University of New South Wales approved such collections.

Mononuclear cells were purified by density gradient centrifugation (Ficoll-Paque; Pharmacia, Uppsala, Sweden) and enriched for CD34⁺ cells with a MACS CD34 isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany) to a purity of 65 to 80% as determined by flow cytometry.

Transduction of primary CD34⁺ cells. Cord blood or peripheral blood CD34⁺ cells were cultured in StemPro-34 medium with supplement (Invitrogen) plus the cytokines interleukin-3, stem cell factor, Flt-3 ligand, and thrombopoietin, each at 20 ng/ml (R&D Systems). CD34⁺ cells were cultured in cytokines at 37°C for 16 to 64 h. For infection, they were resuspended at 5×10^5 /ml in VCM containing various ratios of the LNGFR and LGFP retroviral vectors plus 16 μ g of Polybrene per ml. After 6 h, the cells were resuspended at 2.5×10^5 cells/ml in StemPro-34 medium with cytokines as previously described, cultured for 3 days, and then analyzed by flow cytometry to determine the proportion of cells that expressed each transgene.

Flow cytometry. MACS-enriched CD34⁺ cells from mobilized peripheral blood or cord blood were stained with anti-CD34-phycoerythrin (anti-HPCA2; Becton Dickinson Immunocytometry Systems, San Jose, Calif.) and analyzed with a FACSort flow cytometer (BDIS) on channel FL2 with acquisition of at least 10,000 events. Acquisition control and analysis were performed with CellQuest software (BDIS). Following transduction and culture, TF-1 cells or primary CD34⁺ HPC were stained for NGFR expression with anti-NGFR (Boehringer, Mannheim, Germany), followed by rat anti-mouse PerCP (BDIS). Cellular expression of GFP and NGFR was determined on channels FL1 and FL3, respectively. Single- and double-positive cellular populations were counted by using the gating strategy shown in Fig. S5 of the supplemental material.

Statistical analysis. Proportions have been denoted by $P[x]$, calculated by dividing the number of cells of phenotype x by the total number of viable cells as defined by a forward versus side light scatter region.

The formula defining conditional probability (20) was used to define the proportion of cells counted in a gated subregion by flow cytometry, i.e., $P[x | y] = P[x \text{ and } y]/P[y]$.

Therefore, when cells expressing transgene x are counted in a gate for cells that express transgene y , the proportion of cells that express transgene x within the gated subpopulation y is calculated by dividing the number of cells that express both transgenes (x and y) by the number of cells that express transgene y and is denoted as $P[x | y]$.

The significance of the difference in means for a group of cotransductions that were performed with the same cell source and identical transduction protocols was determined by the paired Student t test (see Fig. S6 of the supplemental material).

Nonlinear regression analysis (Systat version 9; SPSS Inc.) by the quasi-Newton method to minimize the sum of squares was used to find the best-fit parameters for exponential models. Linear regression analysis was used to estimate the slope and intercept for linear models. The error-of-parameter estimates for nonlinear and linear regressions are expressed as 95% confidence intervals (CI).

RESULTS

Effect of viral titer on transduction efficiency. Figure 1 shows the proportion of viable TF-1 cells that were positive for NGFR versus the retroviral titer. At the highest titer, the proportion of transduced cells was 0.83, and it was evident that increasing the titer further would not result in transduction of every cell.

We examined how closely transgene expression fitted an exponential model in which exposure to virus was modeled as

FIG. 1. Relationship between retroviral titer and frequency of transduction. TF1 cells were transduced with LNGFR. The bottom x axis shows the retroviral titer of LNGFR, and the y axis shows the proportion of viable cells that expressed NGFR (●). An exponential model was fitted to the data by the method of least squares. The continuous line shows the two-parameter fitted model ($S = 0.86$, $r = 2.6 \times 10^{-6} \text{ ml} \cdot \text{cell}^{-1}$). The average number of viral particles per cell (m) was calculated by multiplying the retroviral titer (number of viral particles per milliliter) by the coefficient r and is shown on the top x axis. The broken line shows the model that assumes that all cells are susceptible to transduction ($S = 1$). In this case, the estimate for r was $1.85 \times 10^{-6} \text{ ml} \cdot \text{cell}^{-1}$.

a Poisson random variable and only a proportion of cells were susceptible to transduction (see the probability model of susceptibility in the supplemental data).

The proportion of transduced cells = $S(1 - \exp[-m])$, where m is the average number of viral particles that cells are exposed to and S is the proportion of cells that are susceptible to transduction. In this experiment, the viral titer (v), expressed in infectious viral particles per milliliter, was converted to the average number of particles per cell ($m = r \times v$) with r as the proportionality constant. Figure 1 shows a plot of the exponential model with the fitted values of S (0.86 ± 0.05 [95% CI]) and r (2.6 ± 0.39 [95% CI]) as determined by nonlinear regression analysis (assuming $\sigma = 0.025$, $\chi^2 = 22.7$, $P = 0.12$). The bottom x axis shows the viral titer, and the top x axis shows the estimated average number of viral particles per cell. The assumption that all cells were susceptible to transduction ($S = 1$) resulted in a model that did not fit the data as well (see dashed line; $\chi^2 = 56.0$, $P = 0.001$).

Cotransduction of TF-1 cells. TF-1 cells were transduced in duplicate experiments over a 5-h period with the vectors PG13/LNGFR (nine levels ranging from 1.5×10^4 to 1.2×10^6 ivp/ml) and PG13/LGFP (kept constant at 4.9×10^4 ivp/ml). Figure 2 shows the relationship between the proportion of viable cells that were NGFR positive and the proportion of GFP-positive cells that were NGFR positive. There was a linear relationship between these proportions; the line of best fit had a gradient of 0.91 ± 0.03 (95% CI) and passed close to the origin (intercept -0.03 ± 0.02 [95% CI]).

If GFP expression did not influence the susceptibility of TF1 cells to transduction of NGFR, then the proportion of cells

FIG. 2. Proportion of total cells that are NGFR positive (y axis) versus the proportion of GFP-positive cells that are NGFR positive (x axis). Filled circles denote data pairs. The fitted linear equation is shown as a continuous line with the 95% prediction interval (dotted line). The dashed line denotes the expected relationship between the two proportions if transductions were independent, i.e., $P[\text{NGFR}^+ | \text{GFP}^+] = P[\text{NGFR}^+]$. The data below this line of equality indicate that GFP expression facilitates the expression of NGFR.

that were NGFR positive should equal the proportion of GFP-positive cells that were NGFR positive. The dashed line shown in Fig. 2 is the equality of $P[\text{NGFR}]$ and $P[\text{NGFR} | \text{GFP}^+]$, where transgene expression is assumed to be independent. All of the data points were below this line, indicating that GFP expression made it more likely for cells to be transduced with NGFR.

We next investigated whether this permissive effect of GFP expression was statistically significant. Using the same data, we plotted the proportion of GFP-positive cells that were NGFR positive versus the proportion of GFP-negative cells that were NGFR positive (see Fig. S6A of the supplemental material). LNGFR-positive cells were found more frequently in the GFP-

positive cell subset. This increased expression was highly significant (0.52 ± 0.08 versus 0.43 ± 0.07 [$P < 0.001$, paired Student t test]).

It was also possible that NGFR expression had a similar permissive effect on GFP expression. Figure S6B of the supplemental material shows that the proportion of NGFR-positive cells that were GFP positive was higher than the proportion of NGFR-negative cells that were GFP positive (0.152 ± 0.006 versus 0.090 ± 0.005 [$P < 0.001$, paired Student t test]). Thus, cells transduced with one vector had a better than random chance of transduction by the other vector.

In separate experiments, TF-1 cells were transduced in triplicate over a 4-h period with the vector amounts shown in Table S2 of the supplemental data (LNGFR, 3.5×10^4 to 5.4×10^5 ivp/ml; LGFP, 3.4×10^4 to 4.3×10^5 ivp/ml [see supplemental material]). As found previously, cells transduced with one vector had a better than random chance of being transduced by another. Figure 3A shows that the proportion of cells that were NGFR positive (right y axis) was directly proportional to the proportion of GFP-positive cells that were NGFR positive (upper x axis). Shown on the same graph is the proportion of cells that were GFP positive (left y axis) versus the proportion of NGFR-positive cells that were GFP positive (bottom x axis). Both sets of data points were closely correlated to the linear regression (slope = 0.77 ± 0.03 , y intercept = -0.02 ± 0.02 [95% CI]). All of the plotted data points lay below the dashed line of independence ($P[\text{GFP}] = P[\text{GFP} | \text{NGFR}]$ and $P[\text{NGFR}] = P[\text{NGFR} | \text{GFP}]$), indicating that transduction with one vector increased the probability of transduction by the second vector. The gradient of the data shown in Fig. 3A (4-h transduction) was significantly lower than that of Fig. 2 (5-h transduction), suggesting that a longer incubation time resulted in a gradient that was closer to unity.

Figure 3B shows the plot of the product of the proportions of cells that were NGFR and GFP positive versus the proportion of cells that coexpress these transgenes for the same data shown in Fig. 3A. The slope of the regression line ($k \approx 0.76$)

FIG. 3. Cotransduction of TF1. (A) $P[\text{GFP}^+]$ versus $P[\text{GFP}^+ | \text{NGFR}^+]$, left y axis versus bottom x axis; data pairs are denoted by open circles. $P[\text{NGFR}^+]$ versus $P[\text{NGFR}^+ | \text{GFP}^+]$, right y axis versus top x axis; data pairs are denoted by closed circles. The fitted linear equation is shown as a continuous line with the 95% prediction interval (dotted line). The dashed line denotes the expected relationship between the two proportions if transductions were independent. (B) $P[\text{NGFR}^+P[\text{GFP}^+]$ versus $P[\text{NGFR}^+\text{GFP}^+]$. The fitted linear equation is shown as a continuous line with the 95% prediction interval (dotted line). The gradient of the fitted linear equation is similar to that in panel A. The packaging cell line did not influence the ratio of $P[\text{NGFR}^+P[\text{GFP}^+]$ to $P[\text{NGFR}^+\text{GFP}^+]$.

FIG. 4. Cotransduction of cord blood. Left y axis versus bottom x axis; data pairs are denoted by closed circles. $P[NGFR^+]$ versus $P[NGFR^+ | GFP^+]$, right y axis versus top x axis; data pairs are denoted by open circles. The fitted linear equation is shown as a continuous line with the 95% prediction interval (dotted line). The dashed line denotes the expected relationship between the two proportions if transductions were independent ($S = 1$).

was very close to that shown in Fig. 3A. Transductions performed with the PG13 (open circles) and PA317 (closed circles) packaging cell lines are also shown in Fig. 3B. The packaging cell line did not appear to influence the linear regression relationship.

Cotransduction of cord blood CD34⁺ cells. Cord blood CD34⁺ cells were prestimulated with cytokines for 3 days (see Materials and Methods), followed by 6 h of transduction with PG13/LNGFR and PG13/LGFP. Flow cytometric analysis was done after 3 days of culture. Table S3 of the supplemental material shows the retroviral titer and the proportion of cells that expressed NGFR, GFP, or both. Figure 4 shows that the proportion of cells that were NGFR positive (right y axis) was directly proportional to the proportion of GFP-positive cells that were NGFR positive (upper x axis). Shown on the same graph is the proportion of cells that were GFP positive (left y axis) versus the proportion of NGFR-positive cells that were GFP positive (bottom x axis). Both sets of data points were closely correlated to the linear regression (slope = 0.36 ± 0.06 [95% CI]). All of the plotted data points lay below the dashed line of independence ($P[GFP] = P[GFP | NGFR]$ and $P[NGFR] = P[NGFR | GFP]$), indicating that transduction with one vector increased the probability of transduction by the second vector. The slope for cord blood was significantly lower compared to transductions of TF-1 (5-h transduction, $0.91 \pm$

0.03 [95% CI] [Fig. 2]; 4-h transduction, 0.77 ± 0.03 [95% CI] [Fig. 3]).

Cotransduction of mobilized peripheral blood CD34⁺ cells. Mobilized peripheral blood CD34⁺ cells were prestimulated with growth factors for 16, 39, or 64 h before the 6-h transduction protocol was commenced with a mixture of PG13/LGL (1.5×10^5 ivp/ml) and PG13/LNGFR (1.8×10^5 ivp/ml). After 3 days of growth in cytokines, the expression of NGFR and GFP was determined by flow cytometry.

Table 1 shows the results of these experiments. The highest level of transduction for both GFP and NGFR was obtained after 39 h of stimulation with cytokines and was only 1.9 and 4.7%, respectively. The proportion of GFP- or NGFR-positive cells was much higher in gated NGFR- or GFP-positive subsets, 16.5 and 39.9%, respectively. A similar pattern was observed for other time points.

Prior studies with TF1 and cord blood cells demonstrated direct proportionality between the percentage of transgene-positive cells in viable cells and the percentage of transgene-positive cells in a gate defined by expression of the other transgene. A mathematical model of cotransduction experiments presented in the Discussion provides evidence that the proportionality constant, estimated from the gradient (Fig. 2, 3A, and 4), is equal to the proportion of cells that are susceptible to transduction. The proportionality constant was calculated from the ratio of ungated to gated cells for cotransduction of mobilized peripheral blood CD34⁺ cells (Table 1, last column). After 16 h of preincubation, the susceptibility was 6%, and it increased to 12% at 39 h, which indicates that the longer preincubation period had a positive effect on transgene expression because cell susceptibility was highest at this time.

DISCUSSION

The development of techniques to transduce and simultaneously resolve at least two reporter genes by flow cytometry (1, 3, 5, 10) has provided a new investigative tool for methods of rapid screening of high-titer retroviral packaging cell lines (4) and the study of retroviral interference (11, 19). We have cotransduced TF1 cells and cord and mobilized peripheral blood CD34⁺ cells with two marker vectors and shown that cells transduced with one vector had a better than random chance of transduction by the other vector. For identical transduction conditions, the percentage of transduced cells was directly proportional to the percentage of transduced cells within a gate defined by expression of the other transgene (Fig. 2, 3A, and 4). The proportionality constant was less than one and was influenced by transduction times and the target cell population, but not the packaging cell lines, used in this study

TABLE 1. Cotransduction of mobilized peripheral blood CD34⁺ cells^a

Preincubation time (h)	Frequency				Ungated/gated ratio
	Overall		Gated		
	GFP	NGFR	GFP NGFR	NGFR GFP	
16	0.012 (0.001)	0.021 (0.003)	0.197 (0.029)	0.355 (0.063)	0.06 (0.001)
39	0.019 (0.001)	0.047 (0.003)	0.165 (0.028)	0.399 (0.023)	0.118 (0.016)
64	0.011 (0.003)	0.029 (0.001)	0.135 (0.027)	0.356 (0.026)	0.081 (0.009)

^a Frequency is expressed as the average of duplicate experiments (range).

(PG13 and PA317, Fig. 3B). In addition, for a set of transduction conditions, the value of the proportionality constant was not influenced by the choice of transgene used to calculate the ratio (Fig. 3A and 4). We have proposed a simple model to explain these experimental observations.

The probability model of susceptibility (see supporting information for a detailed mathematical derivation) is based on the following assumptions: (i) cells are randomly exposed to a vector; (ii) not all cells are susceptible to vector uptake, integration, and expression (transduction); and (iii) cells exposed to two identical vectors that only differ in their transgene insert will have identical susceptibility if there is negligible interference between these vectors. The result of this model is a mathematical formula for cell susceptibility (S) described by the equation $S = P[A]/P[A | B] = P[B]/P[B | A] = P[A]P[B]/P[AB]$, where $P[A]$, $P[B]$, and $P[AB]$ are the proportions of cells that express transgene A, B, and both A and B, respectively. $P[A | B]$ and $P[B | A]$ are the proportion of B-positive cells that express transgene A and the proportion of A-positive cells that express transgene B, respectively.

The model provides an explanation for the finding that a cell transduced with one vector has a better than random chance of transduction by the second vector. The reason is that cells expressing a transgene are susceptible to transduction and hence if exposed to a second vector are more likely to be also transduced by that vector. The formula shown above predicts that the percentage of transduced cells is directly proportional to the percentage of transduced cells within a gate defined by expression of the other transgene; the proportionality constant is equal to the proportion of cells that are susceptible to transduction. Thus, the susceptibility of TF1 cells and cord blood can be estimated from the gradients of the linear regressions shown in Fig. 2, 3, and 4. TF1 cells incubated for 4 or 5 h with retroviral vectors had susceptibilities of 77% (Fig. 3) and 91% (Fig. 2), respectively. The culture conditions used to transduce cord blood resulted in a susceptibility of 36% (Fig. 4).

The mathematical identity for susceptibility yields three algebraically identical expressions to calculate susceptibility (see formula above), which explains why under identical transduction conditions, the susceptibility could be calculated with either GFP or NGFR. Figure 3B confirms that susceptibility is also calculated by dividing the product of the proportions of cells that are positive for A and B by the proportion of cells that are double positive.

The multiplicity of infection is defined as the ratio of vector particles to cells. Viral titer is usually measured by transduction of a target cell line at limiting vector dilutions. The implicit assumption is that at a low vector concentration, a single target cell will receive not more than one vector particle. The number of transduced cells per milliliter is equal, at most, to the number of vector particles per milliliter. However, the number of transduced cells will also be dependent on cell susceptibility.

The model for susceptibility assumes that exposure to a vector is a random process that can be modeled as a Poisson random variable. Transduction will only occur in susceptible cells that are exposed to virus. Probability of transduction = $S(1 - \exp[-m])$, where m is the average number of vector particles per cell and S is the susceptibility. Thus, as the number of vector particles increases without bound, the probability of transduction will equal the susceptibility, as depicted in Fig. 1.

The model also generates a formula that can be used to estimate the average number of vector particles per cell by eliminating susceptibility (S) from the two expressions shown above. $m_A = -\ln[1 - P(A | B)]$ and $m_B = -\ln[1 - P(B | A)]$, where m_A and m_B are the average numbers of vector A and B particles per cell. This is a useful formula providing an estimate of the average exposure of cells to virus based on cotransduction data. Taken together, formulae for m and S provide a method to estimate the influence of vector titer and cellular factors on the level of transduction.

For example, the transduction levels of mobilized peripheral blood CD34⁺ cells were very low (Table 1) and it is likely that transgene expression was related to low susceptibility (6 to 12%). Because the average exposures of mobilized peripheral blood CD34⁺ cells to the LGFP and LNGFR vectors, calculated with the formula shown above, were 0.18 and 0.46 vector particles per cell, a low viral titer was also a contributing factor. Transduction efficiency would be improved by increasing cell susceptibility and by increasing the titers of the LGFP and LNGFR retroviruses by at least five- and twofold, respectively, to achieve on average at least one virus particle per cell.

In previous investigations, Walker et al. determined that cells could be transduced with two distinct retroviral vectors by simultaneous or sequential transduction protocols (19). They hypothesized that if each virus infected cells independently, then the expected frequency of dual transduction would be equal to the product of the transduction frequency of each virus. When both viruses were added to the same tube, the predicted efficiency was 0.8% (38% of 2%), less than the measured frequency of double-positive cells (1%, see Walker et al., Fig. 5D). This result is consistent with our observation that for experiments in which both vectors are added to the same tube, the dual-transduction frequency is always greater than the product of the individual-transduction frequencies (susceptibility is less than one). This provides evidence that transgene expression was not independent and that cells transduced with one vector had a greater likelihood of transduction by the other.

NIH 3T3 cell-derived packaging cells such as the PA317 and PG13 cells used in this study have been shown to secrete inhibitors that prevent transduction of target cells (8, 18). An alternate explanation to account for the apparent plateau effect observed in Fig. 1 may be the prevention of further transduction by these inhibitors. However, we did not observe a decrease in susceptibility at a high viral titer, indicating a lack of effect on susceptibility (Fig. 2, 3, and 4). For an identical transduction protocol, the estimate of susceptibility based on the plateau value ($S = 0.86 \pm 0.05$ [95% CI]; Fig. 1) was similar to the estimate based on dual-transduction data ($S = 0.91 \pm 0.03$ [95% CI]; Fig. 2).

MacNeill et al. have demonstrated interference at the receptor level between competing amphotropic murine leukemia virus (MLV) vectors when used for simultaneous infection on fibronectin CH-296 (11). There was no interference between an MLV vector and a GALV pseudotyped vector. Therefore, overall transduction could be enhanced by use of PA317 (MLV)- and PG13 (GALV)-packaged vectors.

We did not find a significant difference in susceptibility between TF1 cells transduced with a PA317-packaged vector and those transduced with a PG13-packaged vector (Fig. 3B). We

have calculated susceptibility from MacNeill's dual-transduction data (see Table S4 of the supplemental material). Analysis of that data has shown that the susceptibility of HEL cells transduced with GFP and the B7.1 molecule was 0.86 if both vectors were packaged with PA317 cells and 0.88 if B7.1 was packaged with PG13 cells. Thus, it is possible that the combination of packaging cell lines enhanced transduction by increasing virus delivery at the cell surface but did not enhance the efficiency of vector processing once the vector had gained entry into the cell. These results support a lack of influence of the packaging cell line on susceptibility.

Wider experimental studies are required to test whether the efficiency of intracellular vector processing is the biological basis for enhanced susceptibility. To our knowledge, this is the first report that provides a mathematical interpretation of co-transduction data that results in a formula to calculate the proportion of susceptible cells. The practical utility of this technique is that nonsaturating levels of virus can be used to estimate a theoretical maximum transduction rate for a cell given a specific set of culture conditions. It is also possible to determine whether low transduction efficiency is related to low retroviral delivery at the cell surface or susceptibility of the cell to transduction. Future research to establish the biological basis of susceptibility should include more extended studies of viral delivery mechanisms and establishment of the fate of vectors once they have entered the cell.

The measurement of cell susceptibility with simultaneous vector transduction is useful for developing strategies to optimize gene transfer efficiency. The methodology developed in this study will provide a valuable tool for understanding how retroviral delivery and cellular integration pathways determine the transduction rate.

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REFERENCES

1. Abad, J. L., F. Serrano, A. L. San Roman, R. Delgado, A. Bernad, and M. A. Gonzalez. 2002. Single-step, multiple retroviral transduction of human T cells. *J. Gene Med.* **4**:27–37.
2. Dick, J. E. 2000. Gene therapy turns the corner. *Nat. Med.* **6**:624–626.
3. Giaretta, I., D. Madeo, R. Bonaguro, A. Cappellari, F. Rodeghiero, and P. Giorgio. 2000. A comparative evaluation of gene transfer into blood cells using the same retroviral backbone for independent expression of the EGFP and deltaLNGFR marker genes. *Haematologica* **85**:680–689.
4. Green, B. J., and J. E. Rasko. 2002. Rapid screening for high-titer retroviral packaging cell lines using an in situ fluorescence assay. *Hum. Gene Ther.* **13**:1005–1013.
5. Hawley, T. S., W. G. Telford, A. Ramezani, and R. G. Hawley. 2001. Four-color flow cytometric detection of retrovirally expressed red, yellow, green, and cyan fluorescent proteins. *BioTechniques* **30**:1028–1034.
6. Kitamura, T., T. Tange, T. Terasawa, S. Chiba, T. Kuwaki, K. Miyagawa, Y. F. Piao, K. Miyazono, A. Urabe, and F. Takaku. 1989. Establishment and characterization of a unique human cell line that proliferates dependently on GM-CSF, IL-3, or erythropoietin. *J. Cell. Physiol.* **140**:323–334.
7. Knaan-Shanzer, S., D. Valerio, and V. W. van Beusechem. 1996. Cell cycle state, response to hemopoietic growth factors and retroviral vector-mediated transduction of human hemopoietic stem cells. *Gene Ther.* **3**:323–333.
8. Le Doux, J. M., J. R. Morgan, R. G. Snow, and M. L. Yarmush. 1996. Proteoglycans secreted by packaging cell lines inhibit retrovirus infection. *J. Virol.* **70**:6468–6473.
9. Lewis, P. F., and M. Emerman. 1994. Passage through mitosis is required for oncoretroviruses but not for the human immunodeficiency virus. *J. Virol.* **68**:510–516.
10. Lybarger, L., D. Dempsey, G. H. Patterson, D. W. Piston, S. R. Kain, and R. Chervenak. 1998. Dual-color flow cytometric detection of fluorescent proteins using single-laser (488-nm) excitation. *Cytometry* **31**:147–152.
11. MacNeill, E. C., H. Hanenberg, K. E. Pollok, J. C. van der Loo, M. F. Bierhuizen, G. Wagemaker, and D. A. Williams. 1999. Simultaneous infection with retroviruses pseudotyped with different envelope proteins bypasses viral receptor interference associated with colocalization of gp70 and target cells on fibronectin CH-296. *J. Virol.* **73**:3960–3967.
12. Miller, A. D. 1992. Retroviral vectors. *Curr. Top. Microbiol. Immunol.* **158**:1–24.
13. Miller, A. D., and C. Buttimore. 1986. Redesign of retrovirus packaging cell lines to avoid recombination leading to helper virus production. *Mol. Cell. Biol.* **6**:2895–2902.
14. Miller, A. D., J. V. Garcia, N. von Suhr, C. M. Lynch, C. Wilson, and M. V. Eiden. 1991. Construction and properties of retrovirus packaging cells based on gibbon ape leukemia virus. *J. Virol.* **65**:2220–2224.
15. Miller, D. G., M. A. Adam, and A. D. Miller. 1990. Gene transfer by retrovirus vectors occurs only in cells that are actively replicating at the time of infection. *Mol. Cell. Biol.* **10**:4239–4242.
16. Roe, T., T. C. Reynolds, G. Yu, and P. O. Brown. 1993. Integration of murine leukemia virus DNA depends on mitosis. *EMBO J.* **12**:2099–2108.
17. Romano, G., C. Pacilio, and A. Giordano. 1999. Gene transfer technology in therapy: current applications and future goals. *Stem Cells* **17**:191–202.
18. Seppen, J., S. Barry, G. M. Lam, N. Ramesh, and W. R. Osborne. 2000. Retroviral preparations derived from PA317 packaging cells contain inhibitors that copurify with viral particles and are devoid of viral vector RNA. *Hum. Gene Ther.* **11**:771–775.
19. Walker, P. S., U. R. Hengge, M. C. Udey, I. Aksentijevich, and J. C. Vogel. 1996. Viral interference during simultaneous transduction with two independent helper-free retroviral vectors. *Hum. Gene Ther.* **7**:1131–1138.
20. Walpole, R. E., and R. H. Myers. 1989. 1.6 Conditional probability, p. 26–29. In R. E. Walpole (ed.), *Probability and statistics for engineers and scientists*, 4th ed. Macmillan Publishing Company, New York, N.Y.