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RESEARCH ARTICLE

Functional analysis of gammaretroviral vector transduction by quantitative PCR

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Abstract

Background In a clinical setting of gene therapy, quantitative methods are required to determine recombinant viral titres and transgene mRNA expression, avoiding the use of reporter genes.

Methods We describe procedures based on quantitative polymerase chain reaction (qPCR) designed to assess functional titres of murine leukaemia virus (MLV) vectors, determine proviral copy numbers in transduced cells, and estimate retroviral transgene expression in both target cell lines and mice with transduced chimeric haematopoiesis.

Results Compared to EGFP titration, proviral DNA detection by qPCR was more accurate in assessing the number of infective particles in supernatants, such that average viral titres in terms of proviral copies per cell were two-fold higher. Transgene mRNA expression was directly determined from the vectors used without the need for reporter assays. A new parameter, defined here as the 'transcription index' (TI), served to establish the association between transcribed transgenic mRNA and each proviral insertion. The TI represents the potential expression of every vector or insertion in each cell type, and is thus useful as a control parameter for monitoring preclinical or clinical protocols.

Conclusions The practical use of qPCR is demonstrated as a valuable alternative to reporter genes for the assessment and surveillance of insertion numbers and transgene expression. In combination with protein expression, this approach should be capable of establishing safer therapeutic gene doses, avoiding the potential side effects of high transduction and expression levels.

Keywords gammaretrovirus; quantitative PCR; titration; proviral copies; transgene expression; transcription index

Introduction

The advanced stage of development and availability of murine leukaemia virus (MLV)-based vectors and helper cell lines have promoted their popularity for the gene transfer protocols currently being tested in numerous clinical trials [1–3]. However, the ability to integrate into the host genome, a primary advantage of this gene transfer system, raises concerns on safety, since proviral DNA integrates unspecifically in the host genome, and this could lead to inactivation of essential functions or activation of cell oncogenes by insertion in regulatory regions [4–8]. In addition to risks related to transgene insertion, several unexpected side effects arising from ectopic

nonregulated transgene expression may be observed [9–11]. To achieve the desired therapeutic goals while reducing the potential adverse effects derived from the use of a retroviral vector, the transduction potential for specific cells needs to be carefully assessed, to monitor effective vector copy numbers and transgene expression levels in target cells. Although MLV-based vectors are the system most commonly used for gene delivery, few protocols have described the molecular evaluation of their functional titres or correlations with mRNA and protein expression [12–14]. Quantitative polymerase chain reaction (qPCR) has been used to quantify RNA titres in supernatants obtained from MLV-based vectors for the screening of high producer cell lines, avoiding the need for selectable or reporter genes [12]. However, the total number of viral particles calculated by this method fails to distinguish between infectious and noninfectious virus particles. The quantification of infective HIV-derived vector particles in supernatants and transduced cells based on real-time PCR (qPCR) has been recently reported [15–17]. The functional titration of lentiviral vectors through the determination of proviral DNA copies in transduced cells has been found to more reliably estimate infective viral particles than reporter gene or genomic RNA based titration methods. By comparing retroviral transduction rates, estimated by flow cytometry, and proviral cell insertions, determined by qPCR or Southern blotting, positive correlation was revealed between the number of insertions and protein expression in transduced human primary haematopoietic $CD34^+$ progenitors and the K562 erythroleukaemia cell line [18].

The precise determination of viral transgene events and their correlation with gene expression levels are central to the search for safe gene transfer methods for use in preclinical and clinical gene therapy protocols. This report describes the development of a new strategy based on qPCR, designed to determine functional titres and the efficiency of transgene expression by monocistronic and bicistronic MLV-based vectors in *in vitro* and *in vivo* models. In addition, based on the parameters analyzed, we define a new indicator that reflects the relationship between mRNA transgene expression and the number of proviral insertions.

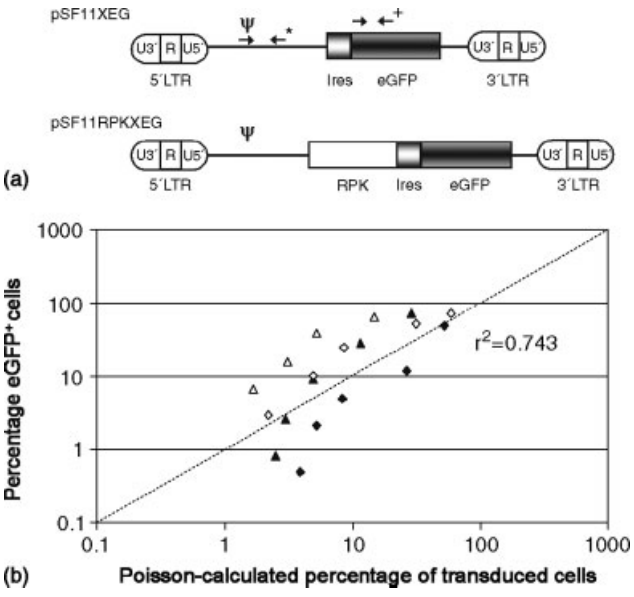

Figure 1. Vectors used and comparative assessment of quantitative PCR and flow cytometry for the quantification of supernatant titres. (a) Schematic representation of the EGFP monocistronic and hRPK/EGFP bicistronic Moloney murine leukaemia virus (MLV)-derived transfer vectors used in this study and positions of the real-time PCR primers. Arrows with plus and asterisk signs represent the binding site positions of the different primers used for DNA and transgenic mRNA quantitative real-time PCR detection, respectively. Each vector genomic-RNA encoding construct contains an Mo-MLV packaging signal (ψ); viral 5'LTR was derived from murine embryonic stem cell virus (MESV); (b), aPS and $EGFP^+$ cells were determined for each cell type, vector supernatant and assay dilution. A comparative analysis of transduced cell percentages obtained by EGFP flow cytometry and those calculated from aPS measurements by Poisson distribution analysis was conducted. Solid triangles (SF11XEG) and diamonds (SF11RPKXEG) represent the supernatant dilutions tested on HeLa cells. Open triangles and diamonds represent dilutions of a similar set of retroviral supernatants tested on 3T3 cells

Materials and methods

Vector constructs and cell lines

The vectors used (Figure 1a) were based on pSF11γ (GenBank accession No. *AJ132035*) [19]. Vector SF11XEG was made by inserting the 5′ untranslated region (UTR) of mRNA of homeodomain Gtx eukaryotic protein as an internal ribosome entry site (IRES) (kindly provided by Dr. V. Mauro, SCRIPs, San Diego, CA, USA) [20] in pSF11γ, upstream from the enhanced green fluorescent protein (EGFP) cDNA sequence. A bicistronic vector SF11RPKXEG was constructed by inserting hRPK cDNA from the pAZ1 expression vector [21], containing full length human pyruvate kinase R (hRPK) cDNA upstream from IRES in SF11XEG.

The 293T cell-based ecotropic (Phoenix-eco) and PG13 packaging cell lines, and HeLa and 3T3, were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY, USA) supplemented with 10% foetal bovine serum (FBS) (Intergen, Purchase, NY, USA). To produce viral supernatants, ecotropic and GALV-pseudotyped SF11XEG and SF11RPKXEG vector packaging cells were established. Supernatants were harvested 24 h after culturing to confluence from packaging cells in Iscove's modified Dulbecco's medium (IMDM) supplemented with 20% FBS.

Animals

Mice aged 8–10 weeks old of strains C57Bl/LY5.1 (B/6.SJL-$CD45^a$-Pep^{3b}, Jackson Laboratory, Bar Harbor, ME, USA) ($CD45.1^+$) and C57BL/6-ly5.2 ($CD45.2^+$) (Jackson Laboratories) were used in the experiments. The animals were kept under constant conditions of

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temperature (22 °C) and light (12 hour light/dark cycle) and routinely screened for pathogens. All experimental procedures were carried out according to Spanish and European regulations (Spanish RD 223/88 and OM 13-10-89 of the Ministry of Agriculture, Food and Fisheries for the protection and use of animals in scientific research; and European convention ETS-123 for the use and protection of vertebrate mammals used in experiments and for other scientific purposes).

Flow cytometry

Protein expression analysis was performed in an EPICS XL flow cytometer (Coulter Electronics, Hialeah, FL, USA) equipped with a 488-nm argon laser. Transduced cells were gated according to scatter criteria. Gate criteria were adjusted according to positive and negative controls.

Protein expression titres and cell line transduction

EGFP titres (transduction units per mL, TU/mL) in viral supernatants were estimated in triplicate by endpoint dilution of cell-free supernatants infecting HeLa or 3T3 cells overnight. After 4 days of culture, the cells were trypsinized and a 2×10^4 cell aliquot was analyzed by flow cytometry to estimate the transduction rate. The remaining cells were used for DNA and mRNA assays. EGFP titres were calculated as previously described [22] using the following formula: titre $= (F \times C_0/V) \times D$; where F is the frequency of $EGFP^+$ cells determined by flow cytometry; C_0 is the total number of infected target cells; V is the inoculum volume; and D is the virus dilution factor.

Quantitative PCR detection of proviral DNA

For quantitative measurements, genomic DNA was isolated from 1×10^5 cells using the Puregene kit (Promega, Madison, WI, USA). To monitor viral infection, the retroviral sequence and unique genomic sequences from human or murine cells were assayed by real-time PCR in an ABI PRISM® 7000 sequence detection system (Applied Biosystems, Foster City, CA, USA). Primers and Taqman MGB probes were designed with the aid of the Primers Express® software package (Applied Biosystems). The specific EGFP PCR product was amplified by the primers: F1EGFP (5′-GTAAACGGCCACAAGTTCAGC) and R1EGFP (5′-TGGTGCAGATGAACTTCAGGG), and detected with the TaqMan MGB probe PEGFP (5′-FAM-CTTGCCGTAGGTGGC-MGB). Amplification of human or murine genomic β-actin sequences was achieved using the primers: F1β-actin (5′-GGGAAATCGTGCGTGACAT) and R1β-actin (5′-CCTGGCCGTCAGGCAG) and amplified sequences detected with the TaqMan probe: P1β-actin (5′-FAM-CTCTTCTCCAGGGAGGA-MGB). Two negative controls were used: one consisting of a sample lacking DNA and the other one comprised of genomic DNA from untransduced cells. Amplification involved one cycle of 95 °C for 10 min, followed by 40 cycles of 95 °C for 30 s and 58 °C for 30 s. β-Actin sequence amplification was performed using identical primers and probe concentrations as used for proviral amplification in one cycle of 95 °C for 10 min, and 50 cycles of 95 °C for 35 s and 55 °C for 30 s. All reactions were carried out in triplicate.

Transgenic mRNA expression

Total RNA extracted from 1×10^5 cells was quantified using the RiboGreen RNA-specific assay (Molecular Probes, Inc., Eugene, OR, USA) and 2.0 μg of total RNA were M-MTLV reverse-transcribed using random primers. To monitor transgenic mRNA expression, the quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) was performed on cDNA from total RNA to amplify a fragment corresponding in the construct to the 5′ UTR region of the mRNA transcripts. qRT-PCR detection was performed using the primer pair: F1ΨRV (5′-GAGGTTCCACCGAGATTTGG) and R1ΨRV (5′-GCAGGCGCAAACATTAGATG), plus the fluorescent Taqman probe P1ΨRV (5′-FAM-TTTCGTGTCTGTCTCTG TCTTTGTGCGTG-TAMRA). Reactions were carried out in triplicate as one cycle of 95 °C for 10 min, and 40 cycles of 95 °C for 30 s and 58 °C for 30 s. Controls in which the reverse transcription step was omitted were included. In addition, negative controls using no DNA and cDNA from untransduced cells as template were run in parallel. Expression of the β-actin gene was used as an internal control. Expression was quantified using a standard curve for the retroviral plasmid (mRNA transgene copies per nanogram of RNA) and relative expression was analyzed following the $E^{-\Delta\Delta ct}$ method [23].

Murine haematopoietic stem cell transduction and bone marrow transplant

Bone marrow (BM) was harvested from C57Bl/LY5.1 ($CD45.1^+$) male mice for the purification of $Lin^-Sca\text{-}1^+$ cells (L^-S^+). BM samples were subjected to red blood cell lysis and then sorted using the murine stem cell MultiSort kit (Miltenyi Biotec, Gladbach, Germany) according to the manufacturer's recommendations. On average, 70–90%-pure populations of L^-S^+ were obtained. For the transduction of fresh L^-S^+, cells were prestimulated for 48 h in IMDM supplemented with 20% FBS, hrIL-11, and mrSCF and then infected on plates coated with 20 μg/cm2 of CH-296 (Retronectin, Takara Shuzo, Otsu, Japan) preloaded with retroviral particles as previously described [24]. Cells were harvested 4 h after the last infection cycle, washed twice in PBA, and 2×10^4 haematopoietic cells/mouse were then injected into the tail vein of lethally

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irradiated (12 Gy in 2 split doses of 6 Gy, 24 h apart) 8- to 12-week-old C57BL/6-ly5.2 (CD45.2+) female mice. Fluorescence-activated cell sorting (FACS) analysis was performed by detecting the CD45.2+ signal and DNA was determined along with mRNA and EGFP expression in BM cells 8 months after transplant.

Results

Provirus quantification and functional DNA titration by qPCR

To determine functional DNA titres (viral particles per mL of supernatant able to transduce target cells; IP/mL), genomic DNA from murine (NIH-3T3) and human (HeLa) transduced cells at several vector dilutions was isolated 4 days after infection. To avoid the detection of possible endogenous retroviral sequences, complementary primers to the EGFP sequence were used to quantify the integrated provirus. Subsequently, IP/mL values were obtained by absolute and relative quantification. Given the half-live of a retroviral particle within the cell is between 5.5 and 7.5 h [25], the analysis performed 4 days after infection avoided the possible amplification of episomal retroviral nucleic acids. Absolute quantification was based on a standard curve prepared using known amounts of the SF11XEG vector plasmid while relative quantification was based on vector EGFP and endogenous β-actin signal detection.

The estimation of absolute DNA titres was achieved by comparing threshold cycle (Ct) values derived from DNA samples to those obtained from a standard curve of known concentrations of plasmid retroviral DNA (1×10^6 to 1×10^1 copies/reaction). Since DNA titration values rely on a reliable standard curve, all points were measured in triplicate and curve r^2 values were always above 0.98. IP/mL values were determined by considering the following parameters according to a previously described procedure [15]: (1) number of copies of the provirus in the PCR reaction; (2) total amount of cellular DNA extracted; (3) volumes and dilution factors of the supernatants; and (4) cell number at the time of infection and final number of cells after 4 days of culture, also used to calculate the average number of proviral insertions per cell.

The relative method involved calculation of DNA titres by direct measurement of the average number of proviral DNA molecules per transduced cell. For this purpose, a standard curve of EGFP/β-actin amplification ratio was prepared using as template proviral DNA from the genomic DNA of a HeLa cell line containing two integrated copies of the proviral EGFP vector (confirmed by Southern blot analysis, data not shown), mixed in proportions of 100 to 0% with a negative genomic HeLa DNA sample. qPCR conducted on EGFP and β-actin sequences served to obtain a standard curve for the DNA mixture. To calculate the average number of proviral sequences per transduced cell (aPS), the EGFP/β-actin amplification ratio (ar) was calculated using the expression: $ar = (E_{pDNA}{}^{Ct\_EGFP}/E_{gDNA}{}^{Ct\_\beta\text{-actin}})$, where E_{pDNA} and E_{gDNA} are the real-time PCR efficiencies of the proviral DNA and genomic DNA amplicons, respectively, and Ct_EGFP and Ct_β-actin are the Cts obtained for each sample. Next, the average number of provirus copies per cell (aPS) was estimated by interpolation of the EGFP/β-actin ratio in the standard curve. Then, using the aPS values obtained, DNA titres were calculated as follows: $IP/mL = (aPS \times C_0/V) \times D$, where C_0 is the total number of infected cells, V is the volume of the supernatant inoculum, and D is the virus dilution factor. As for the absolute quantification method, each point of the standard curve represents the results of assays performed in triplicate ($r^2 > 0.99$).

Table 1 shows the DNA titres determined by both the absolute and relative quantification procedures. The values obtained using both approaches for each supernatant were similar, indicating the accurate determination of the functional DNA titre by both these systems. Average numbers of proviral copies per cell were accordingly also very similar. Relative measurements showed a lower standard deviation than the absolute values, probably due to a normalization effect of β-actin amplification on experimental inter-sample reproducibility.

Comparison of DNA titres and protein expression titres

DNA and EGFP protein expression titres were simultaneously determined in all supernatants. DNA titres were estimated following relative measurement as described above and EGFP expression was determined by flow cytometry. Table 2 compares the DNA and EGFP titres. The EGFP/DNA titre ratio ranged between 0.5 and 3, indicating very similar results for both techniques. To compare the two procedures (protein expression vs. DNA qPCR) independently of the vector and cell line used, the percentage of transduced cells was calculated from the average number of proviral sequences, assuming that provirus transduction follows a Poisson distribution, and compared with the percentage obtained by EGFP detection. A correlation analysis, considering all the dilution data available from each supernatant assayed by the

Table 1. Comparison between absolute and relative DNA titres obtained for supernatants assayed after 4 days

Retroviral construct	Cell line	DNA titre ($\times 10^5$) absolute quantification	DNA titre ($\times 10^5$) relative quantification
SF11XEG	HeLa	1.57 ± 0.41	1.77 ± 0.28
SF11RPKXEG		4.52 ± 0.62	3.35 ± 0.13
SF11XEG	3T3	2.23 ± 0.35	2.04 ± 0.18
SF11RPKXEG		6.59 ± 0.77	5.70 ± 0.27

No significant differences were observed between the absolute and relative quantification of DNA titres ($r^2 = 0.95$; $p > 0.01$).

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Table 2. Comparison between DNA and EGFP titres for supernatants assayed 4 days after transduction

Retroviral construct	Cell line	EGFP titre ($\times 10^5$)	DNA titre ($\times 10^5$) relative quantification	Ratio EGFP/DNA titre
SF11XEG	HeLa	3.24 ± 0.28	1.77 ± 0.28	1.83
SF11RPKXEG		1.97 ± 0.32	3.35 ± 0.13	0.58
SF11XEG	3T3	5.66 ± 0.43	2.04 ± 0.18	2.77
SF11RPKXEG		9.78 ± 0.15	5.70 ± 0.27	1.71

No significant differences were observed between EGFP and relative DNA quantification titres ($r^2 = 0.62$; $p > 0.01$).

two methods (Figure 1b), revealed a clear relationship between both procedures ($r^2 = 0.743$).

Quantification of transgene mRNA expression by qPCR

In addition to the accurate evaluation of infective particles in the viral supernatants and the average number of proviral copies in the target cells, monitoring transgene expression is essential for demonstrating gene functionality in experimental and clinical gene therapy protocols. To evaluate mRNA expression from SF11XEG monocistronic and SF11RPKXEG bicistronic vectors, a qRT-PCR assay was designed to amplify the 5′-UTR mRNA region, common to both vectors, to overcome the possible variation in efficiency caused by different transcript lengths. mRNA expression was analyzed by absolute (copy number per nanogram of total RNA) and relative (normalized to β-actin expression) approaches (Figures 2a and 2b). Since all the samples analyzed showed a strong correlation between absolute and relative measurements, hereafter we only refer to the data obtained using the relative quantification approach.

mRNA transgene expression in the transduced cells was proportional to the vector dilution, which in turn was related to the corresponding aPS value (Figure 2b). In both cell lines, mRNA levels were in agreement with the DNA titres calculated, although murine 3T3 cells showed higher expression than HeLa cells. To compare the mRNA expression efficiency of different constructs in the different cells, we defined a new variable, the transcription index (TI), which reflects the transgenic mRNA synthesized per proviral insertion. This parameter allows the comparison of mRNA expression efficiencies for a given construct in different cell conditions. The TI values calculated for all dilutions derived from a single cell line/vector combination are constant across the proviral range used here (Figures 2c and 2d). Hence, the differences in TI observed for each construct could be attributed to efficiency of the retroviral promoter and/or differences in length and structure between mono- and bicistronic mRNAs, which may also affect their stability. Moreover, given the use of infected cell pools for analysis, effects of the insertion position on expression patterns can be ruled out.

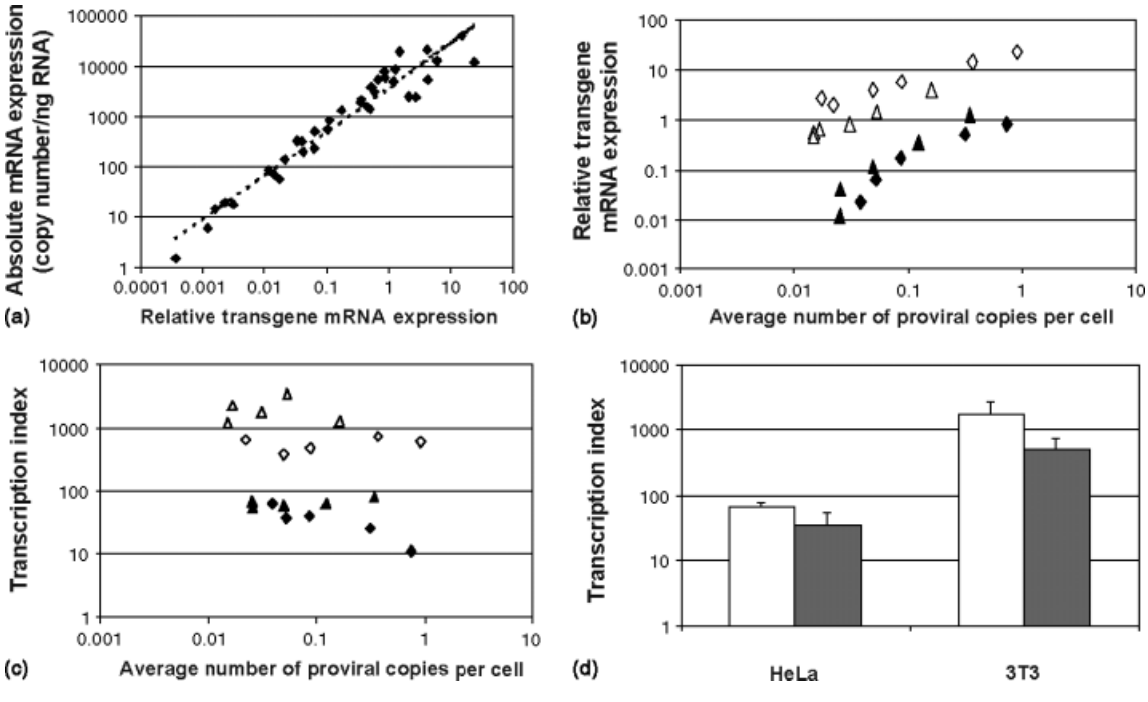

Figure 2. Quantitative analysis of transgenic mRNA expression by real-time PCR. (a) Correlation between absolute and relative measurements of mRNA transgene expression $r^2 = 0.93$. (b) Relative transgenic mRNA expression was compared to the aPS obtained for all the SF11XEG (triangles) and SF11RPKXEG (diamonds) supernatant dilutions tested in HeLa (solid symbols) and 3T3 (open symbols) cells. (c) The number of transgenic mRNAs synthesized per proviral insertion (transcription index = TI) was also explored. The figure shows the TI values calculated from the same set of dilutions compared to aPS values. (d) White and grey bars, representing the SF11XEG and SF11RPKXEG vectors, respectively, show the average TI values obtained from (c) for SF11XEG (white bars) and SF11RPKXEG (grey bars) supernatants, tested in the HeLa and 3T3 cell lines

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In vivo monitoring of average provirus copy numbers and transgene mRNA expression

Finally, average numbers of provirus insertions and mRNA transgene expression were examined after the bone marrow (BM) transplant of lethally irradiated mice with $Lin^{-}Sca\text{-}1^{+}$ haematopoietic progenitor cells transduced with SF11XEG viral supernatants. The number of provirus copies per cell, percentage of $EGFP^{+}$ cells and transgene expression were determined in BM cells 8 months posttransplant. As shown in Figure 3, the transplanted animals showed comparable proviral insertion numbers in the 0.05–0.3 range. As observed *in vitro*, the number of proviral insertions showed correlation with the percentage of $EGFP^{+}$ cells ($p < 0.01$) and mRNA expression ($p < 0.01$) in the hematopoietic compartment evaluated. Again, TI values were similar in all the samples analyzed (Figure 3b: values in parentheses). Moreover, TI values obtained for the BM of the mice were around 200 times lower than values obtained in 3T3 cells. These differences could be explained by improved promoter activity in 3T3 fibroblasts.

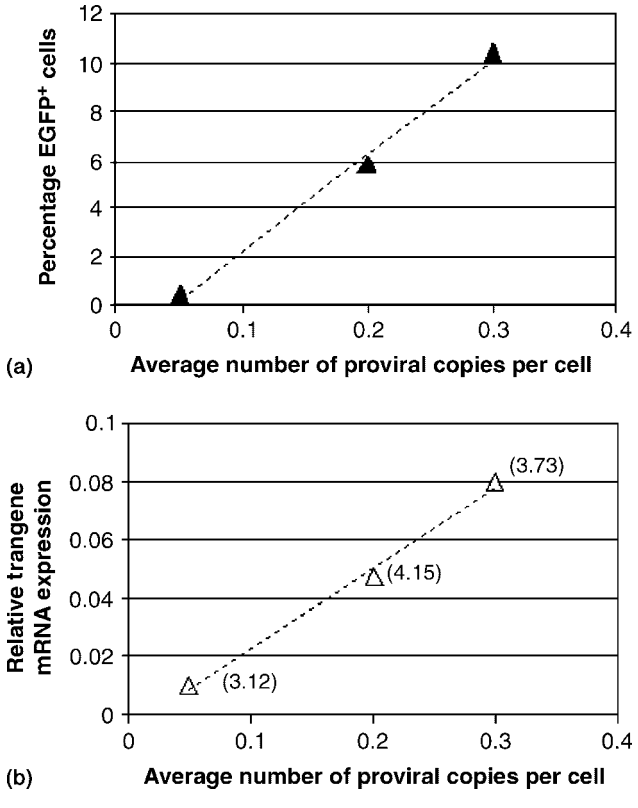

Figure 3. ***In vivo*** **monitoring of aPS and transgene expression in chimeric bone marrow (BM) of transplanted mice. (a) Comparing aPS values and the percentage of $EGFP^{+}$ cells in BM samples from mice transplanted with $Lin^{-}Sca\text{-}1^{+}$ hematopoietic progenitors transduced with SF11XEG (each solid triangle represents a single mouse). (b) aPS values vs. relative transgenic mRNA expression for the same set of BM samples. Numbers in parentheses are the calculated TI values for each mouse**

Discussion

The number of infectious retroviral particles in each supernatant is a key factor that needs to be considered in transduction protocols, to control the amount of vector used in each experiment. Indirect methods have been developed to estimate the number of target cells successfully transduced by the retrovirus based on properties (reporter or selection markers) acquired after transduction [26–28]. Direct quantitative methods, such as measurement of physical particles (electron microscopy) or indispensable components present in the supernatant of all retroviral particles (reverse transcriptase or genomic RNA), detect higher levels of vector particles in retroviral supernatants than those predicted by indirect methods (reporter protein expression or selection markers) [12–14,29,30], since direct methods cannot distinguish infectious from noninfectious viral particles [14,15]. In contrast, indirect methods could underestimate true titres because of a possible reduced capacity to detect all transduced cells [14,30]. Additionally, high multiplicities of infection (MOIs) causing multiple insertions in a cell cannot be detected by indirect methods. This report describes two alternative quantitative PCR procedures capable of quantifying the number of proviral insertion events in transduction trials based on absolute or relative quantification, which could be used to determine the viral particles in a given supernatant able to successfully transduce target cells. Our method combines the high sensitivity shown by real-time PCR (capable of detecting as few as 10 copies of proviral DNA per sample analyzed in our experiments) with an assessment of retroviral insertion events. Although the transcriptional status of proviral insertions is not assessed, the method is able to detect all possible integrations in cells, an important factor to consider in transduction protocols.

To compare the real-time PCR approach with an indirect method, we used gammaretroviral vectors featuring the EGFP reporter gene. Although subtle differences between EGFP and DNA titres were observed in our analysis, these variations were relatively small compared to those reported in real-time PCR estimates made in HIV-derived vector supernatants, in which DNA titres were 10- to 100-fold higher than EGFP titrations [15,16]. These discrepancies could be the consequence of the known differential biological ability to transduce cells in retroviral delivery systems based on both oncoretroviruses or lentiviruses [31,32]. Given the increase in leukemogenesis that occurs with the number of gammaretroviral insertions in gene therapy both in experimental and clinical settings, it is imperative that optimal transduction conditions are defined [8,33,34]. Indeed, the *in vivo* quantitation and monitoring of proviral insertions using the procedures described here would help to predict and reduce the risks of insertional leukemogenesis.

A second and very important consideration in gene transfer and gene therapy studies is the need to

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precisely measure transgene expression levels in target cells. Transgene expression is affected by a variety of factors, including the cell environment, the number of proviral copies in the transduced cells, the insertion position, the specific promoters and regulatory sequences used, the length of mRNA produced from retroviral constructs, etc. [17,20]. In fact, transgene expression has been widely explored by Northern blotting and conventional or semi-quantitative RT-PCR, although the data obtained can only be considered qualitative. The present RNA evaluation method based on real-time PCR accurately measures specific RNA transgene expression levels. Moreover, although functional protein expression is the most accurate way to measure the efficacy of a gene therapy approach, these molecular techniques could be very useful when there are no available reporter genes or when expression is difficult to determine.

Given the accurate qPCR assessment of the number of proviral copies and mRNA copies per cell, we combined both factors to define a new parameter, the transcription index (TI), indicating the number of viral mRNA copies with respect to the number of proviral DNA copies per cell. This ratio provides information on the competence of each construct in a given context. In our experiments, the TI value was constant depending on the expression cassette and cell type such that mRNA expression efficiencies in different experimental designs could be accurately compared. The TI values obtained in the infected murine 3T3 cell line were remarkably higher than those obtained in the infected hematopoietic cells after *in vivo* transplantation. A higher activity of the LTR promoter in 3T3 cells could explain this evidence. However, it is not yet known whether this link between number of proviral copies and mRNA expression levels would be affected by different experimental conditions, thus compromising the predictive significance of the TI. For instance, in conditions of high MOIs, some vector integration events could become transcriptionally unproductive.

Using these quantitative PCR approaches, we were able to monitor *in vivo* the expression of a reporter gene in mouse stem cell transduction and transplantation experiments. Our study on bone marrow specimens demonstrated a high correlation between the number of proviral copies per cell and the percentage of $EGFP^+$ cells. Finally, a strong correlation was also observed between proviral copies per cell and transgene mRNA expression. Hence, through monitoring proviral vector DNA, transgene mRNA expression levels and the TI, information on the haematopoietic dynamics of each transduced cell population was obtained, since biological processes, such as oligoclonal expansion and differentiation, experienced by the original transduced progenitors could be more extensively explored. Other processes such as vector silencing, lineage transgene expression and multiple insertion events could also be accurately assessed. Collectively, our findings indicate that the TI would be a very useful indicator for the routine follow up of gene therapy protocols.

In conclusion, this report describes a set of real-time PCR procedures designed to accurately measure proviral DNA and transgene mRNA derived from Moloney murine leukaemia virus vectors, allowing the assessment of functional DNA titres and the expression potential of vector constructs in specific experimental conditions. The determination of these variables in *in vivo* assays suggests they could provide knowledge on the dynamics of retroviral transduced biological systems and the safety of the current protocols and retroviral delivery systems used in preclinical and clinical gene therapy studies.

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