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Dynamics of transgene expression in a neural stem cell line transduced with lentiviral vectors incorporating the cHS4 insulator

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Abstract

Transplantation of genetically manipulated cells to the central nervous system holds great promise for the treatment of several severe neurological disorders. The success of this strategy relies on sufficient levels of transgene expression after transplantation. This has been difficult to achieve, however, due to transgene silencing. In this study, we transduced the neural stem cell line RN33B with self-inactivating lentiviral vectors and analyzed transgenic expression of green fluorescent protein (GFP) in several different settings both in vitro and after transplantation to the brain. We found that the transgene was affected of silencing both when transduced cells were proliferating and after differentiation. To prevent silencing, the cHS4 insulator was incorporated into the lentiviral vector. We found that a vector carrying the cHS4 insulator was partially protected against differentiation-dependent downregulation in vitro and in vivo. However, in proliferating cells, we found evidence for variegation and positional effects that were not prevented by the cHS4 insulator, suggesting that the mechanism behind silencing in proliferating cells is not the same mechanism influencing differentiation-dependent silencing. Taken together, these findings favor vector optimization as a strategy for achieving efficient ex vivo gene transfer in the central nervous system.

Keywords: Gene therapy; Insulator; cHS4; Ex vivo; Silencing; Green fluorescent protein; Transplantation

Introduction

The concept of transplanting genetically transformed cells into the central nervous system (ex vivo gene therapy) has many potential applications. Severe disorders such as Parkinson's disease, Alzheimer's disease, and Multiple Sclerosis are diseases that in the future may benefit from such a treatment strategy. Although several preclinical studies have been performed in animal models and substantial beneficial outcome has been detected in some cases, the effects of transgene expression have often been small and will be hard to translate to a clinical setting [1–5].

The difficulties several studies have encountered may be attributed to a substantial loss of transgene expression after transplantation [3,6]. Despite the fact that the transplanted cells survive and integrate into the host brain, the level of transgenic protein diminishes shortly after transplantation. There is a substantial lack of knowledge regarding the actual mechanisms that are involved in this process. Histone deacetylation and DNA methylation of the integrated transgenic DNA have been suggested to play a part, but there are no reports that confirm this hypothesis in vivo in the brain [6–8].

We have begun to study the mechanisms of transgene downregulation in brain transplants. In previous reports, we demonstrated that it is possible to mimic the downregulation in an in vitro model and that downregulation is attenuated by treatment with inhibitors of methylation or histone deacetylation [7,9]. This suggests that transgene downregulation might be avoided by optimizing vector design in such a way as to prevent mechanisms such as methylation of the transgene.

In the present study, we used lentiviral vectors to drive expression of green fluorescent protein (GFP) in neural progenitor cells. As a model cell line, we used the conditionally immortalized rat cell line RN33B [10]. This cell line is easily propagated in vitro and therefore allows reliable and reproducible analysis. After transplantation, the RN33B cells have been shown to differentiate into the

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cells of the nervous system and integrate functionally into the host brain [11]. Lentiviral vectors have been suggested to have unique properties that enable them to withstand downregulation of transgene expression [12,13]. The few studies that have used cells transduced with lentiviral vectors as a source for transplantation to the brain have been highly promising in regards to transgene expression relative to other techniques, such as plasmid transfection or onco-retroviral vectors [14–18]. However, it was also evident from these studies that a substantial loss of transgenic expression after grafting occurred [16–18].

To improve the features of the lentiviral vector, we inserted one copy of the 1.2-kb chicken β-globin (cHS4) insulator into the long terminal repeat (LTR). Insulators are *cis*-acting boundary elements that can prevent enhancer–promoter interactions if placed in between these elements and protect transgenic expression cassettes from silencing and positional effects [19]. These effects have been proposed to be separable modalities, but the actual mechanisms are still under debate. Insulators have also been shown to reduce methylation of transgenes and protect transgenes against the influence of surrounding chromatin when used in onco-retroviral vectors [20,21]. In two recent reports, it has also been shown that insulators can be incorporated into lentiviral vectors and that the inclusion of the insulator improves transgene expression [22,23].

Furthermore, recent findings have highlighted the risk of insertion of the provirus into the genome, leading to transformation of the host cells [24,25]. Chromatin insulators have been suggested to decrease this risk by shielding surrounding genes from the internal promoter in the vector and thereby increasing the safety of the vector [26].

Materials and methods

Plasmids

A *Kpn*I–*Xba*I fragment containing the 3′ LTR was isolated from a pHR' plasmid (pHR'CMV.GFP.W), containing the cytomegalovirus promoter (CMV) promoter, the GFP-cDNA, and the woodchuck posttranscriptional regulatory element (WPRE) [27,28]. The fragment was then cloned into the corresponding sites of a pUC18 plasmid. This plasmid was digested in three different ways generating a 1.8-kb fragment (*Eco*RV–*Sca*I), a 0.9-kb fragment (*Sal*I–*Sca*I), and a 1.7-kb fragment (*Sal*I–*Pvu*II). These fragments were religated generating a plasmid (pUC.SIN) containing the same 400 bp deletion, from *Eco*RV to *Pvu*II generating a novel *Bbs*I site, in the U3 region (SIN deletion) as described by Zufferey et al. [29].

The cHS4 insulator was isolated as a 1.2-kb *Sac*I fragment from the plasmid pJC5-4 [30] and inserted in either the 5′–3′ or 3′–5′ orientation in the *Bbs*I site of pUC.SIN (both plasmid and insulator were blunt-ended before ligation) generating plasmids pUC.SIN.INS+ and pUC.SIN.INS−. These plasmids were cut with *Kpn*I–*Xba*I and ligated into the corresponding sites of pHR'CMV.GFP.W, generating two new plasmids pHR'CMV.INS+ and pHR'CMV.INS− (Fig. 1A). By inserting the cHS4 into the 3′ LTR, the integrated provirus will be flanked on both sides by the insulator.

A control plasmid composed of the *Kpn*I–*Xba*I fragment of pUC.SIN ligated into pHR'CMV.GFP.W was also produced (pHR'CMV.SIN).

Vector stock preparation

Lentiviral vectors were produced as previously described [27] and concentrated using ultracentrifugation. Viral stocks were stored at −80°C. To determine titers, two independent methods were used. Particle density was estimated by performing RNA slot blot with a 0.6-kb ^{32}P-labelled DNA fragment of WPRE as probe and the signal was quantified using a phosphoimager (BAS-5000, Fujifilm) with a plasmid containing WPRE as standard [31]. To estimate the concentration of functional transducing units (TU/ml), 293T cells were transduced with serial dilutions of viral stocks. A dilution resulting in less than 15% GFP-positive cells was used for analysis. All viral production and titration were performed simultaneously to minimize variations.

Cell culture

RN33B and HiB5 cells were grown and differentiated as previously reported [7]. For clonal isolation, transduced cells were diluted to <10 cells/ml and 100 μl cell suspension/well was transferred to 96-well plates. Green clones derived from a single cell were selected and expanded. The clonal cultures were initially FACS analyzed when sufficient numbers of cells had been generated. The clones were then further expanded 2–3 passages to obtain sufficient number of cells for FACS sorting and TrichostatinA (TSA) analysis. To study the effect of histone deacetylation on lentiviral transcription, the clonal cultures were exposed to the histone deacetylase inhibitor TrichostatinA (TSA, 0.5 μM, Sigma) for 24 h.

Taqman PCR analysis

To assess the proviral load, we measured the level of integrated lentiviral vectors with a single copy gene as a genomic reference (IL2) using real-time quantitative PCR technique (ABI PRISM 7700, Applied Biosystems, MA).

In brief, 5×10^5 cells were lysed in 18 μl 50 mM Tris–HCl, pH 8.2 (Amresco), 100 mM NaCl (Merck), 5 mM EDTA (Chemicon), and 0.5% SDS (Chemicon). After addition of 2 μl Proteinase K (10 mg/ml, GibcoBRL), samples were incubated for 30 min at 55°C. Water (200 μl) was then added and samples were boiled for 10 min.

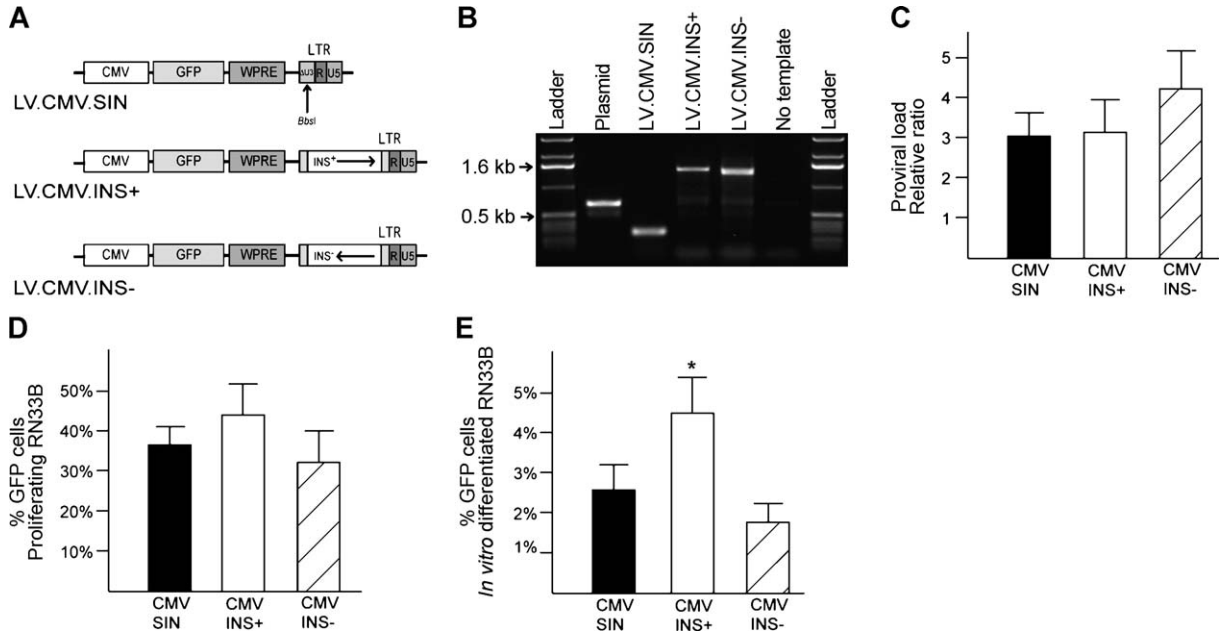

Fig. 1. Transgene expression in RN33B cells. (A) Schematic drawing of the vectors used in this study. The cHS4 insulator was inserted in both directions in the 3′ LTR. (B) PCR analysis of the 5′ LTR. Plasmid DNA (pHR'CMV.SIN) amplifies a full-length LTR (0.6 kb). DNA from RN33B cells transduced with LV.CMV.SIN amplifies, as expected due to the events during reverse transcription when the SIN deletion in the 3′ LTR is duplicated, a smaller fragment (0.2 kb). DNA from RN33B cells transduced with LV.CMV.INS+ and LV.CMV.INS− amplifies a 1.4-kb band, confirming that the full-length cHS4 insulator (1.2 kb) is duplicated into the 5′ LTR. (C) Similar number of proviruses, as measured with Taqman PCR, per cell after transduction with the vectors used in this study ($n = 3$). (D) Similar number of GFP-expressing cells in proliferating RN33B cells after transduction at an MOI of 1 ($n = 5$). (E) Downregulation of transgene expression after 7 days of in vitro differentiation. Significantly more cells expressed GFP when transduced with CMV.INS+ ($n = 5$), * denotes significantly different from other groups, $P < 0.005$, Fishers PLSD, error bars = standard deviation. CMV = human cytomegalovirus promoter, GFP = green fluorescent protein, WPRE = woodchuck posttranscriptional regulatory element, INS+ = cHS4 insulator inserted into the lentiviral vector in sense direction, INS− = cHS4 insulator inserted into the lentiviral vector in anti-sense direction, LTR = long terminal repeat. Ladder = Gibco 1-kb ladder.

Taqman PCR was carried out according to the guidelines in Userbulletin#2, http://www.appliedbiosystems.com. Two microliters of cell extract was used in total volume of 25 μl Taqman universal master mix (Applied Biosystems). Quantification was performed using the comparative C_T method. The clone with the lowest proviral load (RN33B.INS + 6) was assigned a relative value of 1. Primers and probes: lentivector, forward primer, ACTTGAAAGCGAAAGGGAAAC, reverse primer, CACCCATCTCTCTCCTTCTAGCC, probe, Fam-AGCTCTCTCGACGCAGGACTCGGC-Tamra, ratIL2, forward primer, GCCTTGTGTGTTATAAGTAGGAGGC, reverse primer, AGTGCCAATTCGATGATGAGC, probe, Fam-TCTCCTCAGAAATTCCACCACAGTTGCTG-Tamra.

PCR analysis of proviral insulator duplication

PCR amplification using a primer pair that specifically amplifies the 5′ LTR was performed to confirm the presence of the 1.2-kb cHS4 insulator in the 5′ LTR after reverse transcription [32]. Briefly, 2 μl of cell extract (see above) was used to amplify proviral DNA using RedTaq (Sigma) in a total volume of 25 μl. PCR conditions were 94°C 10 min followed by 40 cycles of 94°C 1 min, 63°C 2 min, 72°C 3 min. Primers, 5LTR forward primer, 5′-AAG GGC TAA TTC ACT CCC AA-3′, 5′ LTR reverse primer, 5′-TCGAGAGAGCTCCTCTGGTTT-3′.

Flow cytometric analysis and cell sorting

Cells were trypsinized, washed, and resuspended in Hank's balanced salt solution (HBSS, GibcoBRL) +1% Bovine Serum Albumin (Sigma). A FACSCalibur (Becton Dickinson) was used to measure fluorescence and a FACSVantage (Becton Dickinson) for sorting. The GFP-expressing population was selected by excluding cells overlapping the profile of non-transduced cells. Mean fluorescence unit (MFU) values represent the fluorescent intensity of GFP-expressing cells. Data were analyzed using FlowJo software (Tree Star). FACS analysis was performed in three independent cultures and repeated three times, that is, a total of nine experiments per clone were performed.

Surgical procedures

All animal procedures were conducted in accordance with the regulations of and approved by the Lund University Ethical Committee for Experimental Research in Animals. The rats were housed under 12-h light–dark cycle with ad libitum access to food and water.

On the day of transplantation, cells were trypsinated and resuspended into a single cell suspension in HBSS at a cell density of 100,000 cells/μl. Cells transplanted to adult animals were 4 days before transplantation labelled with ^{3}H-thymidine (1 μCi/ml, Amersham).

Transplantation of RN33B cells to adult and neonatal recipient was performed as previously described [7,33]. Briefly, in the experiment with adult animals, cell suspensions (1 or 3 μl) were bilaterally injected, using a stereotaxic frame, into the striatum of Sprague–Dawley rats (female, 225 g, B and K Universal, Stockholm) at the following coordinates: TB = 0, A = +1.2, L = ±2.5, V = −4.5, −5.0. To evaluate the possible transfer of ^{3}H-thymidine to host cells, transplantation of ^{3}H-thymidine-labelled cells killed by repeated freeze–thaw cycles was performed (n = 4). A total of 53 striata were grafted with 100,000 (n = 28) or 300,000 (n = 25) cells. Animals were sacrificed after 1 week (n = 13, SIN: 4, INS+: 6, INS−: 3) or 3 weeks (n = 14, SIN: 6, INS+: 6, INS−: 2).

In the experiment with neonatal animals, cell suspensions (2 μl) were unilaterally injected at two sites into the cortex and hippocampus (coordinates: A = +0.7, L = −1.9, V = −1.5, −2.9) using a stereotaxic frame with a neonatal device. Each clone (n = 12) was transplanted to at least four neonatal animals. Animals were sacrificed 4 weeks after transplantation.

Histology

At sacrifice, the brains were perfused with 150–300 ml 4% PFA (Merck) in 0.1 M phosphate buffer pH 7.4 (Merck), coronally sectioned (30 μm, neonatal or 40 μm, adult) on a freezing microtome, and collected in five or eight series. Sections were quenched in 3% H_2O_2 for 10 min and then stained for GFP with a primary polyclonal chicken antibody (1:5000, overnight incubation at room temperature, Chemicon), a secondary biotinylated rabbit anti-chicken (1:400, 2 h at room temperature, Jackson), and visualized with an ABC-complex (Vector), using 0.5 mg/ml 3,3-diaminobenzidine (Sigma) as a chromogen. One series of GFP-stained sections from the adult transplantation study was processed for autoradiography by dipping in photographic emulsion (K5, Illford Scientific Products) to reveal ^{3}H-thymidine-labelled cells. After 4 weeks of exposure, sections were developed in Kodak D19 developer. The specificity of the GFP stainings was confirmed by directly analyzing GFP autofluorescence.

To quantify the number of GFP-expressing cells, we counted the labelled cells in a full series of sections and estimated the number of cells using Abercrombie's formula [34]. In the experiment where cells were transplanted to neonatal animal, the clones where categorized as silenced (−, no detectable GFP expression), low expression (+, GFP expression detected in a few cells per animal), medium expression (++, GFP expression detected in approximately 10–100 cells/animal), or high expression (+++, GFP expression detected in a vast amount of cells per animal).

Statistical analysis

Data from the FACS analysis were subjected to analysis of variance (ANOVA) using StatView software (Abacus Concepts) followed by a Fisher PLSD post hoc test when appropriate. In the case of comparing GFP-positive transplants, a χ^2 test was performed. The coefficient of variation (CV) value was calculated as the standard deviation as a percentage of the mean.

Results

Vector functionality and transduction of neural progenitor cells

We constructed two lentiviral vectors expressing GFP under the human cytomegalovirus promoter (CMV) and carrying the 1.2-kb cHS4 insulator in the U3 region of the 3′ LTR in either sense or anti-sense direction (LV.CMV.INS+, LV.CMV.INS−, Fig. 1A). Due to the events during reverse transcription, this vector design generates an integrated provirus that is flanked on both sides by the insulator. As a control vector, we used a self-inactivating lentiviral vector (LV.CMV.SIN) [29]. When viral vectors were produced, similar numbers of viral particles were generated when using insulator constructs as compared to the control LV.CMV.SIN vector (RNA slot blot data: LV.CMV.SIN, 2.2×10^{11} particle units/ml, LV.CMV.INS+, 3.5×10^{11}, LV.CMV.INS−, 2.6×10^{11}). However, the functional titer was markedly reduced for the insulator vectors. Serial dilution titration on 239T cells revealed a loss of almost 90% of the functional particles (CMV.SIN, 1.2×10^8 transducing units/ml, CMV.INS+, 1.1×10^7, CMV.INS−, 1.0×10^7). This finding was repeated in several vector preparations and is consistent with another report where reduced titer has been found after incorporation of insulator elements in lentiviral vectors [23]. By performing a PCR reaction that specifically amplifies the 5′ LTR, we confirmed that the 1.2-kb cHS4 insulator was duplicated after reverse transcription (Fig. 1B).

Based on the functional titers, we transduced the neural progenitor cell line RN33B at multiplicity of infection (MOI) of 1. Taqman PCR confirmed that the proviral load was similar after transduction with either insulator or control vectors (relative proviral load: LV.CMV.SIN, 3.1 ± 0.6, LV.CMV.INS+, 3.2 ± 0.8, LV.CMV.INS−, 4.3 ± 0.9, Fig. 1C). FACS analysis of the transduced cultures revealed a similar number of GFP-positive cells and similar levels of GFP expression in LV.CMV.SIN and LV.CMV.INS+ transduced cells, while we found a slightly lower level of expression in the LV.CMV.INS population (Fig. 1D—Percentage of GFP$^+$ cells: LV.CMV.SIN, 37 ± 8, LV.CMV.INS+, 44 ± 9, LV.CMV.INS−, 32 ± 8, mean fluorescence

units in the GFP-positive population (MFU): LV.CMV.SIN, 186 ± 25, LV.CMV.INS+, 209 ± 19, LV.CMV.INS−, 115 ± 41*, * denotes significantly different from other groups, $P < 0.01$, Fishers PLSD).

Transgene downregulation in vitro in neural progenitor cells

RN33B cells are conditionally immortalized with the SV40 temperature-sensitive allele such that these cells proliferate when cultured at 33°C in vitro and differentiate at 37°C in a defined media. We have recently reported that this system can be used as a model to study transgene downregulation in neural cells [7]. When we applied this model to the lentiviral transduced cultures, we found a robust downregulation of transgene expression in all three populations. However, the remaining number of GFP-expressing cells was higher in the population transduced with LV.CMV.INS+ as compared to the LV.CMV.SIN and LV.CMV.INS− populations (Fig. 1E—Percentage of GFP cells: LV.CMV.SIN, 2.6 ± 0.6, LV.CMV.INS+, 4.5 ± 0.9*, LV.CMV.INS−, 1.8 ± 0.5. * denotes significantly different from other groups, $P < 0.005$, Fishers PLSD), indicating a protective effect of the cHS4 insulator against transgene silencing during in vitro differentiation. The data also confirm previous findings where the boundary effect of the insulator has been reported to be orientation or position dependent [20,21,35]. No difference in proliferation and differentiation capacity was detected between cells transduced with insulator containing vectors and control vector. The finding that an increased number of cells remained GFP-expressing when cells were transduced with the LV.CMV.INS+ vector was confirmed twice using different vector batches. Similar results were obtained when performing the same experiment using the conditionally immortalized HiB5 cell line (data not shown).

Variegation in single-cell-derived clones

To further characterize the ability of the cHS4 insulator to protect against downregulation, we isolated 18 single-cell-derived, clonal populations from GFP-transduced RN33B cells (six clones for each viral construct). FACS analysis revealed no difference between colonies regarding fluorescence intensity of proliferating cells (ANOVA, $P = 0.57$). When the FACS profiles of the various clones were plotted, both narrow peaks and broad, left shifted profiles (indicating transgene variegation) were found at a similar frequency for each of the three viral constructs (Fig. 2A). In some of the colonies, only a minority of the cells were expressing GFP. To test that the transgene variegation was actually a result of transgene silencing in the clones and not a result of mutations in the transgene or due to impure colonies, we performed two experiments.

To begin with, we treated the clones with a histone deacetylase inhibitor (TrichostatinA, TSA). All clones were responsive to TSA, and after 24 h of treatment, GFP expression was increased and the clones displayed a right-shifted FACS profile (Fig. 2B). Even clones that were almost completely silent displayed a homogenous population of GFP-expressing cells after 24 h of TSA treatment, indicating that the GFP transgene remained capable of functional transcription and had not been mutated in any functional way.

Next, we FACS-sorted three of the clones that were subject to transgene variegation (RN33B.INS+4, RN33B.INS−5, and RN33B.SIN5) into two populations based on their GFP expression. The sorting parameters were set so that after sorting there would be two distinct populations: nonexpressing cells and highly expressing cells (Fig. 2C). We then cultured these populations separately and FACS-analyzed them every second week. Variegation was rapidly restored in the populations (Fig. 2C). The GFP expression in the nonexpressing populations slightly increased and the GFP expression in the highly expressing populations declined. After 8 weeks, the FACS profiles of the separated population were very similar. As seen in the FACS profiles (Fig. 2C), this long-term culturing led to an almost complete transgene silencing. It was possible to restore transgene expression also at this late time point by treating the cells with TSA (data not shown). Single-cell-derived clones were also produced from HiB5 cells and the same pattern of narrow and broad FACS profiles was found (data not shown).

Positional effects and proviral load

The differences in transgene expression level found between the various clones can be explained either by a difference in proviral copy number or by influence from the integration site. We performed Taqman PCR on the RN33B clones to measure proviral load (Table 1). To evaluate positional effects, the average level of expression per copy and the coefficient of variation (CV) were calculated. There were no significant differences between the various groups of clones with regards to average level of expression per copy (MFU: SIN, 109 ± 88; INS+, 66 ± 53; INS−, 86 ± 152; $P = 0.78$ ANOVA). The CV value (standard deviation/mean) can be used as an indication of positional effects. As reported earlier, a CV greater than 50% indicates major positional effects while a CV lower than 50% indicates position-independent expression [36]. The CV values for the various groups of clones were SIN, 81%; INS+, 81%; INS−, 176%. CV values of this magnitude indicate major positional effects. Notably, we were unable to detect any effects of the cHS4 insulator against variegation or positional effects in proliferating cells.

Transgene downregulation in vitro in RN33B clones

We applied the in vitro downregulation model to the 18 RN33B clones. All clones downregulated transgene expression to a great extent (Table 1). Due to different baselines in the clones at the start of the analysis, an unbiased analysis is

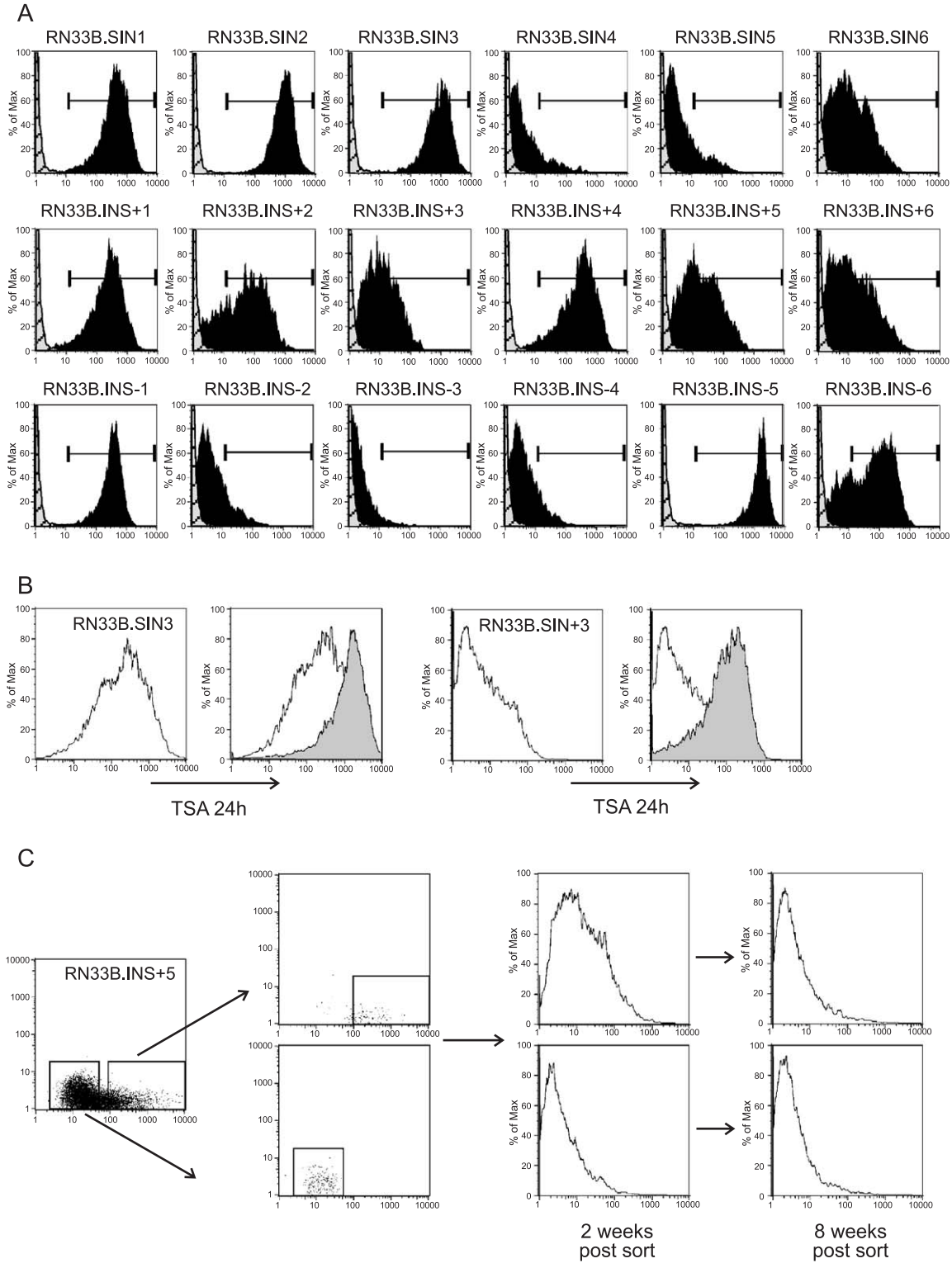

Fig. 2. Analysis of transgene variegation. (A) FACS profiles of 18 RN33B clones carrying the different constructs used in this study. Notice the similar frequency of narrow and broad peaks (*y*-axis, number of cells, *x*-axis: fluorescent intensity, FU, logarithmic scale, grey-hatched profiles represent non-transduced RN33B cells, horizontal bar indicates the GFP-expressing population). (B) Treatment with TSA reverses transgene variegation in RN33B clones (*y*-axis, number of cells, *x*-axis, fluorescent intensity, FU, logarithmic scale). (C) GFP-positive and -negative cells of the clone RN33B.INS + 5 were FACS-sorted (dot plots) and then cultured separately. Variegation was reestablished with time, although no overlap with regards to GFP expression existed immediately after the sort (dot-plots: *y*-axis, forward scatter, *x*-axis, fluorescent intensity, FU, logarithmic scale, FACS profiles: *y*-axis, number of cells, *x*-axis, fluorescent intensity, FU, logarithmic scale).

Table 1
Analysis of GFP expression and proviral load in RN33B clones

Clone	Proliferating cells				In vitro-differentiated cells	
	MFU[a]	Percentage of GFP cells[a]	Proviral load[b]	MFU/copy	MFU[a]	Percentage of GFP cells[a]
RN33B.INS + 1	393	96	5,4	73	84	13
RN33B.INS + 2	192	79	10,5	18	30	21
RN33B.INS + 3	44	51	1,1	40	–	1
RN33B.INS + 4	484	97	3,2	151	51	7
RN33B.INS + 5	81	62	6,0	14	–	0
RN33B.INS + 6	98	49	1,0	98	–	2
RN33B.INS-1	186	79	18,0	10	–	0
RN33B.INS-2	46	18	3,2	14	–	0
RN33B.INS-3	48	5	1,6	30	–	0
RN33B.INS-4	35	24	2,2	16	–	0
RN33B.INS-5	2094	99	5,3	395	122	36
RN33B.INS-6	351	99	6,7	52	–	1
RN33B.SIN1	523	97	3,9	134	41	19
RN33B.SIN2	1303	100	6,6	197	26	15
RN33B.SIN3	1367	99	6,3	217	57	51
RN33B.SIN4	102	21	4,3	24	–	2
RN33B.SIN5	59	20	4,6	13	–	2
RN33B.SIN6	66	42	1,0	66	–	1

[a] Each sample represents an average of nine measurements.
[b] Estimated using Taqman PCR.

problematic. Three of the clones (RN33B.SIN2, RN33B.SIN3, and RN33B INS-5) expressed at very high levels (1303–2094 MFU) compared to the other clones (35–523 MFU, Table 1). When analyzing these three clones, it was possible to detect GFP after 7 days of differentiation. However, the GFP levels were very low (26–122 MFU) and were found only in a fraction of the cells (15–51%). The other LV.CMV.SIN and LV.CMV.INS− clones were with one exception almost completely silenced (<3% GFP expressing cells). The one clone that still expressed GFP (RN33B.SIN1) expressed GFP at a low level (8% compared to proliferating cells) in only a portion of the cells (19% compared to 97% in proliferating cells). However, the LV.CMV.INS+ clones seemed to be at least partially protected against downregulation. Three of the clones still expressed GFP after 7 days (11–21% compared to proliferating cells) in a portion of the cells (7–21%).

We decided to apply a filter where a clone was defined as silenced if less than 5% of analyzed cells were expressing GFP or if the fluorescent intensity (as measured by FACS) had decreased by more than 90% after 7 days of differentiation when compared to proliferating cells. In the context of this definition, all six LV.CMV.SIN clones and all six LV.CMV.INS− clones were defined as silenced while only three out of the six INS+ clones meeting this criteria ($P < 0.05$, χ^2).

Transgene downregulation after transplantation to the adult brain

We transplanted three of the clones to adult rat brain to confirm our in vitro results and to estimate the degree of downregulation in vivo. The clones RN33B.SIN1, RN33B.INS + 1, and RN33B.INS − 6 were chosen based not only on their similar GFP expression when cultured in a proliferative state (MFU: 523, 393, and 351, respectively), but also on the fact that RN33B.SIN1 and RN33B.INS-6 were silenced in vitro according to our definitions while RN33B.INS + 1 was not silenced (see above).

We monitored the survival and migration of cells by ^{3}H-thymidine labelling at 1- and 3-week post-grafting. All clones behaved in a similar way with regards to migration of cells as well as survival quantified using unbiased stereological methods (^{3}H-thymidine-labelled cells: 1-week survival; RN33B.SIN1, 54,000 ± 30,720; RN33B.INS + 1, 50,190 ± 25,790; RN33B.INS − 6, 65,330 ± 49,650; 3-week survival, RN33B.SIN1, 36,610 ± 14,684; RN33B.INS + 1, 70,124 ± 8,336; RN33B.INS − 6, 54,071 ± 23,991; 100.000 cells transplanted, ANOVA $P = 0.51$, Figs. 3A and C). Control experiments using dead cells confirmed previous findings using RN33B cells as a source for transplantation, where no background or transfer of ^{3}H-thymidine could be detected [37]. At 1-week post-grafting, around half of the transplants contained a few GFP-expressing cells near the core of the transplant (Table 2). At 3 weeks post-grafting, a significant difference (χ^2: $P < 0.02$) was found between the different clones. The GFP-positive cells had disappeared in RN33B.SIN.1 and RN33B.INS-6 transplants but not in RN33B.INS + 1 transplants (Figs. 3B and D and Table 2). A common feature at both time points was that GFP expression was found only in the core of the transplants and mainly cells with an immature morphology could be identified as GFP-positive (Fig. 3B). We transplanted either 100,000 or 300,000 cells to each striatum, results were

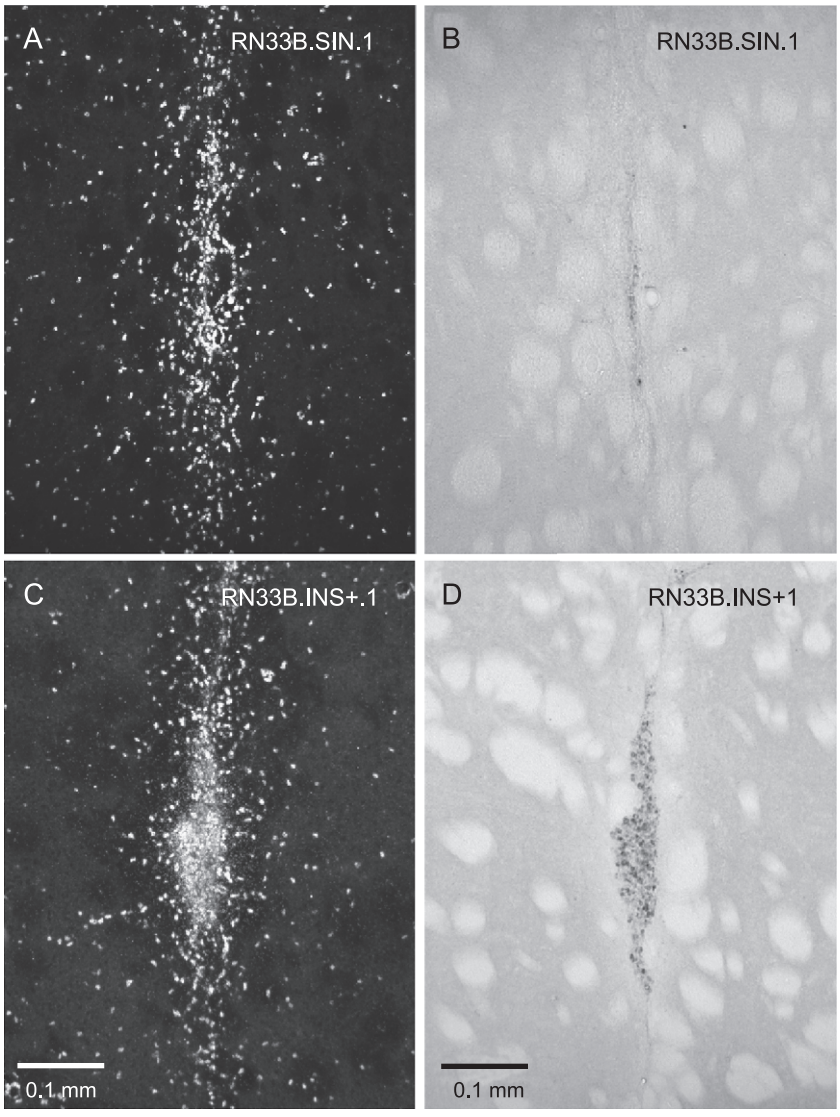

Fig. 3. GFP expression in RN33B cells transplanted to adult rats. (A and C) Dark field images of ^{3}H-thymidine-labelled cells co-labelled with GFP transplanted to the rat striatum. A similar integration and survival of RN33B.INS + 1 and RN33B.SIN1 was detected. (B and D) Corresponding sections immunostained for only GFP. Notice the core of the transplant in B (RN33b.INS + 1) that was still expressing GFP. Scale bar represents 0.1 mm.

similar in the two experimental settings and the results were therefore pooled with regards to GFP expression. The mean number of positive cells was 415 ± 484 in the seven expressing transplants found after 1 week, and 362 ± 366 cells after 3 weeks. In the two other clones (RN33B.SIN1 and RN33B.INS−6), a smaller number of GFP expressing cells were found (RN33B.SIN1, 136 ± 101 cells; RN33B.INS−6, 60 ± 7 cells).

Table 2
GFP-expressing transplants[a] in adult hosts

Clone	1 week	3 weeks
RN33B.INS + 1	7/10 (415 ± 484 cells)[b]	5/12* (362 ± 366 cells)
RN33B.INS − 6	4/6 (60 ± 7 cells)	0/4
RN33B.SIN1	3/8 (136 ± 101 cells)	0/12

[a] A transplant was defined as GFP-expressing when more than 10 clearly stained cells could be identified in a series of sections.
[b] The number of GFP-positive cells was estimated using Abercrombie's formula and is presented as mean ± SD.
* $P < 0.02$ (χ^2).

Transgene expression after transplantation to the neonatal brain

Although transplantation of the three clones into the adult brain indicated that the findings in the in vitro model could be applied to the in vivo situation, the results can be interpreted in several ways. It may actually be so that the integration site in the INS + 1 clone is particularly beneficial, regardless of the presence of insulators, and hereby bias our interpretation. Furthermore, the cells that had migrated out into the host tissue and further differentiated did not

Table 3
GFP expression after transplantation to neonatal hosts

	−[a]	+[a]	++[a]	+++[a]
INS + clones		RN33B.INS + 3	RN33B.INS + 1	RN33B.INS + 4
		RN33B.INS + 6	RN33B.INS + 2	RN33B INS + 5
SIN clones	RN33B.SIN1	RN33B.SIN2	RN33B.SIN4	
	RN33B.SIN3	RN33B.SIN6	RN33B.SIN5	

[a] Specimens were scored blindly on a four-graded scale (see Materials and methods).

express GFP, making any kind of phenotypical analysis impossible. We therefore transplanted the six RN33B.SIN and six RN33B.INS clones to the neonatal rat brain. Transplantation to the neonatal brain has a limitation in that the ^{3}H-thymidine label is transferred to a larger extent to the host brain cells because they divide more compared to adult recipients and hereby not allowing a quantitative analysis. Studies in our laboratory have revealed that transgene downregulation may not be as severe in neonatal transplantation. In fact, when a pool of RN33B cells transduced with a lentiviral vector was transplanted to neonates, substantial numbers of GFP-expressing cells of all cellular subtypes present in the CNS were found [33].

At 4 weeks after transplantation, animals were sacrificed and GFP expression analyzed. Clones were categorized from GFP expression as silenced (−), low expression (+), medium expression (++), or high expression (+++). There was a clear tendency toward higher GFP expression in insulator clones (Table 3). Two of the RN33B.INS+ clones were rated according to the highest score (+++), while a

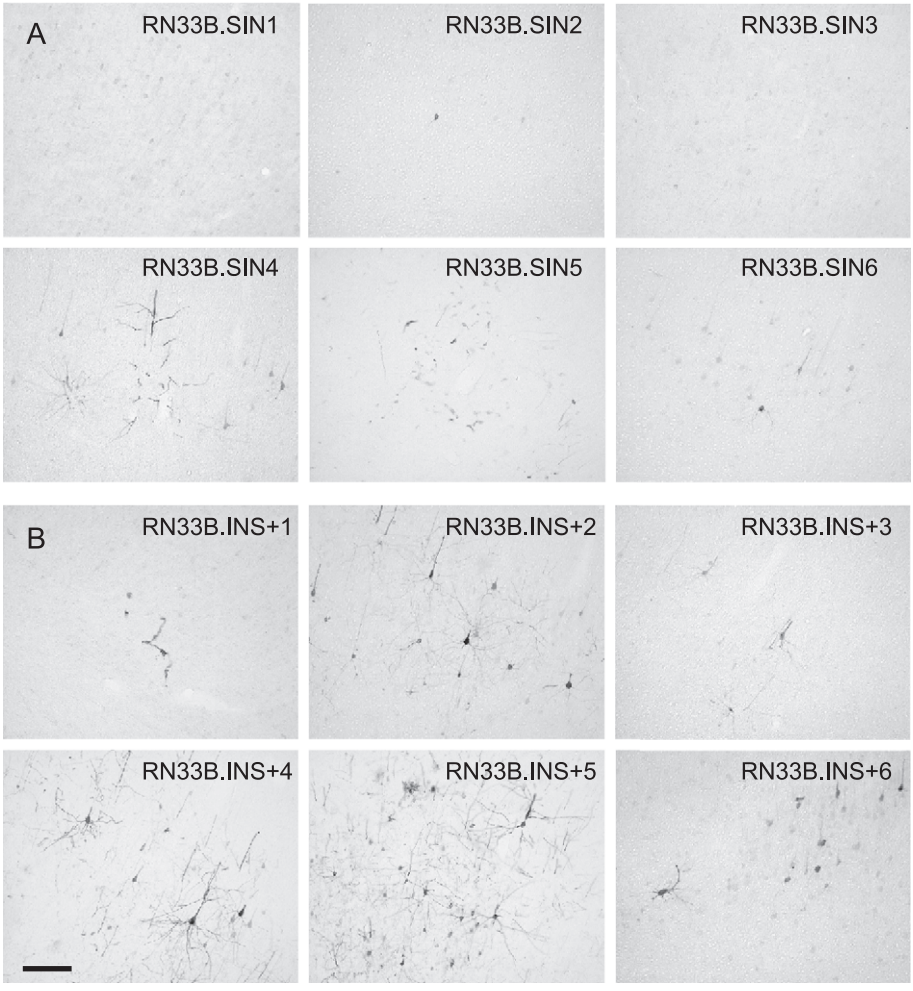

Fig. 4. GFP expression in RN33B cells transplanted to neonatal rats. (A and B) GFP expression in the cortex of rats that were transplanted with the various clones at postnatal day 1 and sacrificed 4 weeks later. GFP expression was found more frequently when RN33B.INS+ clones were transplanted. Scale bar represents 0.1 mm.

complete lack of GFP expression (−) was not found in any RN33B.INS+ clone. The SIN clones expressed GFP less frequently and two of the clones actually completely lacked GFP expression (Table 3, Fig. 4). Some of these differences may be explained by different survival of the various clones. However, RN33B.SIN1 survived equally well as RN33B.INS + 1 when transplanted to the adult brain, suggesting that the lack of GFP expression when transplanted both to neonatal and adult brain was actually due to transgene downregulation. In neonatal animals, the different clones expressed in both immature and mature cells (Fig. 4). Interestingly, there was a difference in the proportion of different phenotypes expressing GFP produced by each clone, suggesting that the proviral integration site influenced what cell type the transgene was expressed in (Fig. 4).

Discussion

The studies reported here show that inclusion of the cHS4 insulator into the LTRs of a lentiviral vector improved transgene expression after differentiation of neural stem cells in vitro and in vivo after transplantation. However, we also detected signs of silencing in proliferating RN33B cells that were not prevented by the cHS4 insulator. This suggests that there are multiple mechanisms of silencing that act on the transgene.

A number of mechanisms may contribute to downregulation. Methylation of the transgenic DNA and histone acetylation has in several reports been shown to be involved in this process (for a review, see e.g., Ref. [6]), but other non-characterized mechanisms are likely to contribute to silencing [6]. The data presented here suggest at least two separate mechanisms influencing transgene downregulation. First, we demonstrate that when RN33B cells proliferated, the transgene was subjected to variegation. This is an indicator of silencing and is associated with deacetylation of the transgene [19]. The clones derived in this study were all responsive to TSA, an inhibitor of deacetylation, suggesting that deacetylation is indeed associated with variegation. Furthermore, in an experiment where we FACS sorted clones into high- and low-expressing populations, we found that the transgene variegation is reversible and that a complete reversal took place after only 4–8 weeks in culture, depending on the clone. Most notably, the cHS4 insulator had no effect on variegation in proliferating cells in vitro. Secondly, when RN33B cells were differentiated, either in vitro or in vivo after transplantation, transgene expression was rapidly and almost completely downregulated. The cHS4 insulator increased the probability that the transgene was expressed after differentiation, suggesting that the mechanism behind differentiation-dependent silencing is different than the mechanism responsible for variegation found in proliferating cells. This has important implications for future vector design. Today, a vector that is developed for ex vivo gene transfer is optimized in proliferating cells. Our data suggest that a different approach is necessary and that models that include differentiation should be used. Interestingly, Klug et al. [38] have reported that when hematopoietic stem cells were transduced with a retroviral vector, a similar separation of silencing at multiple stages was found. This indicates that silencing of transgene expression may share features in cells from different lineages.

The finding of positional effects on transgene expression, in proliferating RN33B cells, is not unexpected. However, the mechanisms behind the positional effects are not clear. It is possible that the transgene is either actively shut off at certain integration sites by proteins recognizing foreign DNA sequences or that the transgene is passively influenced by the surrounding chromatin [39]. In proliferating non-clonal transduced populations, only 30% of the cells were expressing GFP, although the proviral load was 3-fold higher in these populations as compared to the clones with the lowest proviral load (quantified using Taqman PCR, Fig. 1B, Table 1), indicating that transgene expression only occurs from a minority of integrated transgenes. It should be noted that when the various clones were expanded, we initially isolated and selected for active transgenes by only expanding GFP-expressing clones. Interestingly, there seems to be a discrepancy between GFP expression in vitro and GFP expression after transplantation (Tables 1 and 3). A high level of GFP expression in vitro in the various clones did not correlate with a high GFP expression after transplantation to the neonatal brain. This suggests that the positional effects are altered after transplantation, most likely due to a reorganization of the chromatin.

The finding that transplanted clones expressed GFP in different proportions of subtypes of cells (Fig. 4) supports the idea of a so-called ‘bystander’ effect. Accordingly, the transgene is integrated just adjacent to a gene that is active specifically in this subtype of cells and thus activated passively due to its location in the genome. The result of this type of positional effect would not be unravelled unless the cell differentiates into the specific phenotype. This would explain the differences between the proportions of cell types found after grafting of polyclonal GFP-transduced RN33B cells compared to the clones studied here [33].

It is not clear as to what extent the use of promoter influences downregulation. We have in all experiments presented here used a human CMV promoter to drive expression. It has been reported that this promoter is not as active in nonproliferating cells as in dividing cells, and it has been suggested that it has a neuronal tropism [40,41]. The fact that the CMV promoter is cell cycle dependent may to some part explain the finding of variegation in the various clones as well as the restoration of similar FACS profiles after the sorting experiment. However, we detect that transgene variegation occurs in a temporal fashion over weeks, suggesting that other mechanisms than mitosis-dependent regulation are active. It has been reported that the CMV promoter is more prone to transgene variegation when compared to endogenous promoters such as the elongation

factor 1α-promoter [42]. We have in our laboratory used lentiviral vectors carrying the elongation factor 1α-promoter, as well as other promoters, to transduce RN33B cells, and we have in all cases been able to detect transgene variegation (Jakobsson, unpublished data). Furthermore, there are several reports using various promoters that all are strongly affected by transgene downregulation [7,16]. Interestingly, when lentiviral vectors carrying a hCMV promoter were injected directly into the brain, and thus transducing fully differentiated cells, expression was stable over long periods of time (>6 months) at high levels [43]. The transduced cells were not only mostly fully differentiated neurons, but also astrocytes and other glial cells [43,44].

In a preliminary parallel study (J.J. unpublished data), the vectors described in the present study were injected at similar numbers into the brain of adult rats. Quantification, using unbiased stereological methods, of GFP-expressing cells 3 weeks after transduction revealed no difference in the number of GFP-expressing cells when the insulator-containing vectors were used. This finding further strengthens the interpretation that in neural cells, the insulator can act against silencing when cells are differentiating, for example, after transplantation, but has neither effect on transduction into fully differentiated cells nor in proliferating cells.

Many of the clones analyzed in this study seem to have a relatively high copy number as estimated by Taqman PCR (Tables 1 and 3). Given the fact that recent reports suggest a link between high level of proviral integration and mutational effects, this is potentially important [25]. Furthermore, the establishment of transgenic animals using lentiviral germline transduction indicates that a single copy is not sufficient to maintain expression throughout differentiation [13]. This suggests that lentiviral vectors are relatively inefficient in retaining transgene expression after differentiation and that it would be of value to develop more effective vectors to reduce the proviral load.

Insulators are, in addition to their ability prevent transgene downregulation, defined by their enhancer blocking function. This feature confers increased safety to the vectors. In the present study, the level of GFP expression in proliferating CMV.INS+ clones ranged over only 349 MFU while the range was 2059 and 1308 MFU in CMV.INS− and CMV.SIN, respectively (Table 2). One interpretation of this result is that the insulator protects the proviral expression cassette from enhancer activity and that would be important for the development of integrating vectors for gene therapy. The cHS4 insulator has been functionally mapped, and experiments using the different functional units of the insulator in the vectors may shed some light on the mechanisms [45].

In adult animals, downregulation of transgene expression is more pronounced than in neonates. The reasons for this discrepancy are unclear. What is evident from the experiment involving transplantation of the RN33B.INS + 1 clone as well as other findings in our laboratory is that when cells start to migrate out into the host tissue, transgene expression is silenced very effectively [17]. In the study by Ericson et al., primary astrocytes were transduced with lentiviral vectors and transplanted. In this study, a proportion of the cells in the core of the transplant expressed the transgene for a prolonged period of time (6 weeks). As to why astrocytes were more resistant to downregulation than RN33B cells, we can only speculate, but it may be that the differentiation process after transplantation is less extensive in these cells leading to less remodelling of the chromatin compared to RN33B cells. However, when the astrocytes migrated out into the host tissue, transgene expression was silenced in them as well, supporting the idea of a differentiation-associated downregulation.

Conclusion

The findings presented here support a differentiation-dependent model for transgene downregulation in brain transplants. When a cell differentiates following transplantation, the chromatin is remodelled and transgenes are effectively shut-off either through a passive 'bystander' effect or by active mechanisms acting on foreign sequences. In some instances, a transgene avoids downregulation, and this may be due to the integration site being activated by surrounding genes that have been activated during differentiation. Flanking the transgene with the cHS4 insulator significantly increased the probability for a transgene to be expressed after differentiation. This indicates that it may be possible to avoid downregulation by modifying the vector. Improving the vector construct by inclusion of *cis*-acting elements that protect against downregulation or targeting the integration of the vector to genes that are active in differentiated cells may greatly improve transgene expression after transplantation.

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